Survivin Expression in Childhood Medulloblastomas: A Possible Diagnostic and Prognostic Marker

BELA BODEY1,2, VIVIAN BODEY1,2, STUART E. SIEGEL2,3 and HANS E. KAISER4

1Department of Pathology and 2Department of Pediatrics, University of Southern California, Keck School of Medicine, Los Angeles; 2Childrens Center for Cancer and Blood Diseases, Childrens Hospital Los Angeles, Los Angeles, CA; 4Department of Pathology, School of Medicine, University of Maryland, Baltimore, MD, U.S.A. and Department of General and Experimental Pathology, University of Vienna, Vienna, Austria

Abstract. Survivin is a member of the inhibitor of apoptosis gene family that is expressed in embryonic tissues during human ontogenesis and most human malignancies, but it is not present in the majority of normal adult tissues. Survivin is also a chromosomal passenger protein required for physiological cell division. Survivin blocks apoptosis, via its BIR domain, by either directly or indirectly blocking the function of the members of the caspase cascade. The expression of this apoptosis inhibitor protein in medulloblastomas (MEDs) was examined for the first time. During the immunohistochemical study, a sensitive, four-step, alkaline phosphatase conjugated antigen detection technique was employed. The results did, in fact, demonstrate the presence of survivin in 10 to 50 per cent of medulloblastoma (MED) cells with medium intensity immunoreactivity (+ +, B) in this neuroectodermal brain tumor. These results indicate that survivin is probably not only a diagnostic marker, but also an important prognostic marker for MEDs/PNETs and may be useful in the future grading of malignancy in MEDs, much as grading is done today for astrocytomas (ASTRs). Furthermore, the almost exclusive neoplastic expression of survivin will allow development of new antineoplastic, immunotherapeutic strategies.

Survivin

Survivin, a protein that inhibits apoptosis and regulates cell division (1), was discovered in 1997 as an anti-apoptotic protein on the basis of its baculovirus inhibitor of apoptosis repeat (BIR) domain (2). It was recently identified by hybridization screening of human genomic libraries with the cDNA of a factor Xa receptor, effector cell protease receptor-1 (EPR-1). The survivin gene has been found to span 15 kb and is located on chromosome 17 at band q25. Its expression is highly cell cycle-regulated and is detectable in the nucleus selectively at the G2/M-phase (3). Transcription of survivin has even been shown to be directly repressed by p53, another cell cycle checkpoint-regulating protein that induces apoptosis (4). Doxorubicin employment for the treatment of acute lymphoblastic leukemia (ALL) results in accumulation of wild-type p53, resulting in an increased down-regulation of survivin, depletion of cells in the G2/M-phase of the cell cycle and increased apoptosis. Survivin also plays a role in cell cycle progression as demonstrated when it was disrupted by antisense targeting in HeLa cells, resulting in spontaneous apoptosis and aberrant mitosis (5) and an increase in caspase-3 activity (3). Survivin is required for cell division in vivo as well.

Survivin belongs to the Inhibitor of Apoptosis Protein (IAP) family and, as such, is pathologically over-expressed in most human malignancies and functions at the cross-roads of cell death and cell cycle progression. Survivin has actually been detected in most human neoplasms. Survivin, thus, appears to play a significant role in regulating apoptosis at cell cycle checkpoints. Survivin controls a checkpoint associated with chromosome segregation and cell division. Specifically, survivin inhibits apoptosis via its BIR domain by either directly or indirectly interfering with the function of the caspases (6), which are responsible for inducing apoptosis.

The structure of the survivin protein is closely related to its function as an inhibitor of apoptosis. The amino terminal portion of survivin consists of three α helices and three β-sheets, which closely resemble the BIR domain that is part of the IAP family (2,7). It is the BIR domain of the IAP family that is involved in the function of these proteins as inhibitors of apoptosis (8). Survivin was shown to exist as a dimer with the two BIR domains forming a "bow-tie" shape, but how the dimeric structure is involved in the inhibition of apoptosis and cytokinesis requires further observations (7).

Correspondence to: Bela Bodey, 8000-1 Canby Avenue, Reseda, CA 91335, U.S.A. e-mail: Bodey18@aol.com

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The structure of survivin has been compared to another member of the IAP family, XIAP, which contains three BIR domains (9). XIAP inhibits caspase-3 and caspase-7 via a linker region between the first two BIR domains. It also binds to and inhibits caspase-9 through its third BIR (BIR3) domain. The BIR domain of survivin appears to be closely related to the three-dimensional structure of the BIR3 domain of XIAP, which suggests that survivin may bind to caspase-9 (10). In fact, the survivin-caspase-9 complex has been shown to have an effect on the mitotic apparatus, which allowed for caspase-9-dependent apoptosis to occur (11). Specifically, the interaction between survivin and caspase-9 led to the loss of phosphorylation at threonine 34 on the T34A mutant of survivin, resulting in the dissociation of an immunoprecipitable survivin-caspase-9 complex on the mitotic apparatus.

Interestingly, the coding strand of the survivin gene is entirely complementary (antisense) to a previously characterized gene encoding the effector cell protease receptor-1 (EPR-1) (12). Separate promoters, oriented in opposing directions, control the expression of EPR-1 and survivin, wherein transcripts produced for one of these appear to inhibit the expression of the other through an antisense RNA-based mechanism (12). It remains to be determined whether any of these gene loci are directly involved in genetic alterations found in neoplastically transformed cells. Ambrosini and co-workers (12) also discovered that the down-regulation of survivin by forced expression of EPR-1 increased apoptosis and inhibited growth of transformed cells.

Direct binding between survivin and the members of the caspase cascade has not been confirmed, but survivin may also inhibit caspase activity indirectly. There is evidence of an indirect regulation of caspase activity by survivin in the mitochondrial pathway of apoptosis. When Fas is stimulated in a cell culture, survivin has been found to interact with Cdk4, which releases p21 to complex with caspase-3, which is the initial step in the inactivation of caspase-3 in the mitochondria (13,14). While several reports also demonstrate that purified survivin directly binds to caspase-3 and inhibits its activity in vitro (11,15,16), at best, the mechanism of caspase-3 inhibition by survivin remains, nonetheless, controversial.

**Childhood Medulloblastoma**

Tumors of the central nervous system (CNS) represent the second most frequent malignancy in children under 15 years of age, yet are the commonest cause of death. Most adult brain tumors are supratentorial malignant gliomas, whereas the most common malignant pediatric brain tumor is the cerebellar primitive neuroectodermal tumor (medulloblastoma - MED). MED is a non-glial intrinsic malignancy of the brain. As we reported in one of our articles, this common primary, childhood, cerebellar-located malignancy was named MED by Bailey and Cushing (17) based on the brain developmental theory of Schaper (18), who described the presence of “apolar, indifferent cells in the external granular layer of cerebellum” and named them as "medulloblasts" or the common neural stem cells. Despite a number of morphological, histochemical, ultrastructural (transmission electron microscopic-TEM and scanning electron microscopic-SEM) and in vitro observations, evidence for the real existence of the hypothetical "medulloblast" is still lacking (19). In the great majority of cases, three differentiated cell types are found in childhood MEDs: neurons, glia and mesodermal structures (i.e. muscle cells). As we reported, because of the presence of multiple differentiated cell types, these tumors were named after a postulated cerebellar stem cell, the "medulloblast", which would give rise to the differentiated cells found in the tumors. A group of researchers at the Massachusetts Institute of Technology (MIT), USA, described a cell line with the properties expected of the postulated medulloblast (20). The rat cerebellar cell line (named ST15A) expressed an intermediate filament, nestin, that is characteristic of neuroepithelial stem cells. ST15A cells can differentiate, gaining either neuronal or glial properties. At the same time, several clonal cells can also differentiate into muscle cells. These in vitro results suggest that a single neuroectodermal cell can give rise to the different cell types found in MED. Immunocytochemical observations also demonstrated the expression of nestin in human MED tissue and in a MED-derived cell line. Both the properties of the ST15A cell line and the expression of nestin in MED support a neuroectodermal stem cell origin for this childhood tumor.

In a recent retrospective study, Brazilian authors reported the epidemiological and histopathological analysis of 623 primary childhood tumors of CNS occurring during the period 1990 to 1996 (21). In this period, 3318 biopsies of CNS were analyzed. In this total were included 623 pediatric tumors (18.7%). The age of the patients ranged from 5 months to 15 years, 325 tumors occurred in males and 298 in females. The majority affected the posterior fossa. The majority of childhood neoplasias were of glial origin (n=277). The most frequent tumors included: ASTRs (27.9%), MEDs (9.95%), craniopharyngiomas (5.93%), ependymomas (4.97%) and GBMs (3.37%). In another retrospective study, MED represented approximately 20% of all primary brain tumors. The vast majority of them are sporadic. Familial medulloblastoma is very rare, with only a few cases having been reported worldwide; most were observed in siblings of the same sex (22).

In the last decade, there has been increasing recognition of polyphenotypic high-grade malignancies in non-CNS tumor literature. Some of these tumors have been regarded as variants of primitive neuroectodermal tumor (PNET) or
as extrarenal malignant rhabdoid tumors (MRTs). Genetically, PNETs belong to a family of pediatric neoplasms of the CNS that are composed predominantly of primitive neuroepithelial cells. These tumors derive from a carcinogenic alteration of pluripotent neural crest cells, caused by a balanced reciprocal translocation t(11;22) (q24;q12). Among the different CNS PNET, those arising in the posterior fossa (i.e. medulloblastomas) are prototypical of this group of brain tumors. The basic cell biology of PNET is not completely understood, but recent studies of human PNET biopsies and cell lines derived from them demonstrate that neoplastic cells in human PNET recapitulate many of the phenotypic properties of immature CNS neurons or their progenitors. Based on these findings, it has been possible to develop several animal models of human PNET that will enhance efforts to gain fundamental insights into the induction and progression of PNET (23).

In addition, these animal model systems will enable emerging development of new gene engineering biotherapies to be targeted specifically for human PNET.

Scarpelli and co-workers (24) have been exploring data from a set of 14 cases of MED to evaluate whether quantitative image analysis might suggest evidence for the existence of lower and higher grade lesions. MED smears were stained with toluidine blue. For each case, 50 nuclei were measured and a number of densitometric features extracted. The results identified the existence of two subgroups in the observed MED cases, as lower and higher grade groups. A plot of the total optical density versus nuclear area suggested this subclassification. Two nuclear texture features—the number of pixels with the same optical density value occurring consecutively in the nucleus and the proportion of pixels in the high optical density range—divided the cases into the same subgroups. The employment of a clustering algorithm established two clusters that corresponded to that subclassification. Two nuclear texture features—versus nuclear area suggested this subclassification. Two nuclear texture features—the number of pixels with the same optical density value occurring consecutively in the nucleus and the proportion of pixels in the high optical density range—divided the cases into the same subgroups. The employment of a clustering algorithm established two clusters that corresponded to that subgrouping, except for one case. Discriminant analysis gave an identical classification, with the misplaced case having a borderline discriminant function score. An unsupervised learning algorithm based on an adaptive distance metric formed two clusters and assigned the borderline case to the low-grade subgroup. The grouping obtained by quantitative analysis was only partially related to the grade of nuclear atypia subjectively evaluated. The quantitative analysis of the observed MEDs provided a means of detecting differences in the nuclear size and texture, which allowed the classification of cases into two subgroups.

MED is the most common PNET in children, but it is very rare in adults. An isochromosome for the long arms of 17, i(17q), is found in about 30% of pediatric cases. Cytogenetic studies in adults are very scarce; only six cases have been described cytogenetically: three cases had normal karyotype, two were studied partially and another presented only two clonal structural anomalies: del(9)(q12) and del(11)(q22). A group of researchers from Spain studied the chromosomes from MED in a 27-year-old woman and found one hypotetraploid stemline with clonal alterations (25). In the structural anomalies, chromosomes 3, 9, 12, and i(17q) were involved. Chromosome 9 presented a deletion in the long arm, del(9)(q13), with consequent loss of the 9q13-->qter region. This anomaly was similar to one found in a previous case. It has been suggested that the partial loss of the long arm of chromosome 9 may be a characteristic change of adult MED.

The specific aims of this study were: 1) to identify the expression of survivin in MEDs/PNETs, since it has not been previously demonstrated; 2) since survivin has been shown to be useful in the grading of malignancy of astrocytomas, we wondered if this were also possible for MED since the grading of MED is currently nonexistent; and finally, 3) we are always looking for prognostic markers for MED/PNET and we believed that survivin might be a significant one.

Materials and Methods

Tissues and tissue handling. In this immunohistochemical study, we employed formalin-fixed, paraffin-embedded tissue sections of human primary childhood medulloblastomas (DAKO Corporation, Carpinteria, CA, USA). The diagnoses of the specific subtypes of astrocytomas used as positive tissue controls were established according to WHO guidelines for the classification of glioma by a clinical neuropathologist (26-30). Technical details of the immunohistochemical techniques used in this study have already been elaborated by other investigators (31-38) and in the studies published by our group (39-45).

Monoclonal antibody. Anti-survivin rabbit polyclonal antibody (Cat. #RB-1629-P1; Labvision, Fremont, CA 94539, USA). Immunoreactivity: cytoplasmic.

Staining of formalin-fixed and paraffin-embedded tissue sections requires an antigen retrieval technique, employing preliminary boiling in 10mM citrate buffer, pH 6.0 (NeoMarkers, Cat. # AP-9003) for 10-20 minutes, followed by cooling at room temperature for 20 minutes.

Immunohistochemical controls. In order to ensure the specificity of the anti-survivin antibody used in this study, we tested the immunoreactivity of several normal human control tissues including: brain, adrenal, heart, stomach, small intestine, large intestine, liver, kidney, pancreas, lung, testis, ovary, uterus, prostate, thyroid and spleen, all included in one checkerboard multitissue block (DAKO Corporation; code # T1065) (46,47). Several postnatal human thymic specimens were also used as negative and positive tissue controls. A number of neoplastically transformed tissues, including astrocytoma, malignant melanoma and lung cancer tissues, represented the positive tissue controls. Additional controls for all tissues included: 1) omission of the primary, anti-survivin polyclonal antibody; and 2) utilization of only the enzymatic developer solution to detect the presence of endogenous alkaline phosphatase activity; and 3) utilization of MOPC 21 mouse myeloma IgG1 (ICN) as a replacement for the primary antibody to determine non-specific myeloma protein binding to the antigenic epitopes of the screened tissues.
Immunohistochemical evaluation. Qualitative and quantitative evaluation of the percent of antigen-positive cells and the intensity of survivin immunostaining were conducted using a light microscope (Olympus, Japan) counting 100-200 cells. Artifacts were avoided, while, on the other hand, morphologically characteristic areas were sought out. The presence of neoplastically transformed MED/PNET cells with heterogeneous IPs, the endothelial elements of small blood vessels, tumor infiltrating leukocytes and macrophages (the host’s immunological effector cells) required careful qualitative assessment. Non-vascular elements were also examined, but only morphologically distinct MED/PNET cells were scored.

Quantitative evaluation (48): (++++) over 90% of the total cell number are positive; (+++) 50% to 90% of the total cell number are positive; (++) 10% to 50% of the total cell number are positive; (+) 1% to 10% of the total cell number are positive; (+) under 1% of the total cell number are positive; (−) negative.

Qualitative evaluation (48): (A) very intense red staining; (B) strong red staining; (C) light red staining; (D) negative staining.

Results

In this immunohistochemical study, we observed the presence and tissue localization of survivin, employing the specific, mouse, anti-human MoAB against it in MEDs/PNETs. The primary immunoreactivity demonstrated a distribution pattern in 10 to 50 per cent of MED/PNET. The neoplastically transformed cells showed a medium intensity immunostaining (+, B) in the tumor tissue (Figure 1).

Discussion

Our immunohistochemical study identified cytoplasmic expression of survivin in 10 to 50 per cent of the MEDs/PNETs that were explored.

During human development, survivin is expressed in fetal lung, heart, liver, kidney and gastrointestinal tract, and in fetal tissues where apoptosis occurs, such as the stem cell layer of stratified epithelia, endocrine pancreas and thymic medulla (49). In all of these studies, survivin was not found in normal adult tissues, but it has been reported by some to be expressed in bone marrow (50) and the keratinocytes of the basal layer of the skin (51). These findings suggest that the cell division and anti-apoptosis functions of survivin are important not only during early development, but also during cancer progression as well.

While survivin has been detected in most neoplasms, it is not by any means expressed in all tumors. For example, low-grade non-Hodgkin’s lymphomas, which are known for their activation of another type of anti-apoptotic gene, Bcl-2 (52), rarely express this IAP family protein (2). These lymphomas are also tumors with very low growth fractions, a characteristic that could have bearing on the apparent cell cycle-dependent expression of survivin. Moreover, even within a given type of malignancy, heterogeneity in survivin expression may be observed. Immunohistochemical assessments of survivin expression in tumors where immuno-intensity, percentage immunopositivity, or both have been measured for purposes of segregating survivin-negative from survivin-positive (survivin low from high) tumors suggest that expression of survivin (or higher levels of survivin expression) is associated with worse clinical outcome or other unfavorable prognostic features in neuroblastomas, colon and gastric cancers (2,12,49,53). Although preliminary, assessments of survivin expression may be of prognostic significance for patients with some types of neoplasms.

Survivin has been shown to be expressed by neoplasms originating from different cell lineages. There is also cumulative...
evidence that spontaneous immune response against survivin-derived epitopes may occur. Katoh and co-workers used RT-PCR, Western blot analysis and immunohistochemistry to show that survivin is widely expressed by gliomas, meningiomas and schwannomas, both in vitro and in vivo (54). Their data indicate that survivin may serve as an attractive target for immunotherapies designed for brain tumors.

Survivin expression is considered an important prognostic factor of a variety of tumors. Kajiwara and co-workers (55) investigated 43 astrocytic tumors (8 diffuse astrocytomas; 15 anaplastic astrocytomas; 20 glioblastomas) for survivin mRNA expression employing reverse transcriptase-polymerase chain reaction amplification assay. Thirty-four out of 43 (79.1%) astrocytic tumors expressed survivin, with the distributions specifically including 3 out of 8 (37.5%) diffuse astrocytomas, 13 out of 15 (86.7%) anaplastic astrocytomas, 18 out of 20 (90.0%) glioblastomas. Expression of survivin \( (p=0.0057) \) and EGFR \( (p=0.0112) \) was significantly associated with the malignancy grade of astrocytic tumors. It was found that patients with survivin-positive astrocytic tumors had significantly shorter overall survival times compared with patients who had survivin-negative tumors \( (p=0.0271) \). The authors concluded that survivin expression in astrocytic tumors varies with the grade of histological malignancy and may play an important role in the oncogenesis and progression of astrocytic tumors. These data thus suggest that survivin also has great potential as a target in antineoplastic biological therapy of astrocytic tumors.

Our results indicate that survivin is indeed present in MEDs and PNETs. This marker may be of further use in the grading of malignancy for MED/PNET, but such grading will require the use of many more markers. It is our hope that such grading, since it is evident in ASTR to have beneficial effects for prognosis and treatment, will be a possibility in the future for medulloblastomas. As such, the survivin protein could be concluded to be a defining diagnostic marker for MED/PNET. Moreover, survivin as a prognostic marker can also possibly be incorporated into therapy options, which is implicitly indicative of the importance survivin could have. Survivin may, thus, prove to be a useful tool in prognosis and in possible immunotherapy options.

References
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