Novel Tankyrase-related Gene Detected with Meningioma-specific Sera[1]

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ABSTRACT

In many meningiomas, alterations of chromosome 22 can be found, and the *NF2* (neurofibromatosis type 2) gene, in particular, is of great interest as a putative gene involved in meningioma. Because the *NF2* gene is not mutated in all meningiomas, additional genes may be involved. Instead of looking for alterations directly at the DNA level, we used the immune response of meningioma patients to identify immunogenic antigens that may be associated with the disease. We screened a fetal brain cDNA expression library with sera pools from different patients bearing meningioma classified according to the three WHO grades, using the serological identification of antigens by recombinant expression cloning immunological screening method. Here, we report the finding of a new tankyrase-related protein. We found 16 overlapping clones with homologies to tankyrase when we screened the library with the common-type meningioma sera pool and 2 such clones when we screened the library with the atypical meningioma sera. The anaplastic meningioma sera did not identify any tankyrase-related clones. We tested some of the newly identified clones with 13 single sera, 6 of which (37.5%) reacted positively with the tankyrase-related clones. In addition, we screened the tankyrase-related clone with six sera pools from individuals without obvious disease. Although 1 of 24 (4.2%) normal sera reacted with the tankyrase-related clone, we found a striking difference in the frequency of reactivity to this clone by sera from patients bearing tumors corresponding to the three WHO meningioma grades; common-type sera was the most frequently reactive. Northern blot analysis demonstrates expression of the novel tankyrase gene in two common-type meningiomas from patients with immune response.

INTRODUCTION

Meningiomas are tumors of the meninges, the coverings of brain and spinal cord. They account for about 20% of all adult intracranial neoplasms. The WHO distinguishes three meningioma types. The most frequent meningioma, the common-type meningioma, is a slow-growing tumor (WHO grade I). Atypical meningioma (WHO grade II) accounts for about 8% of meningiomas and is characterized by increased cellularity and mitotic activity. The third group, anaplastic meningioma (WHO grade III), is characterized by obvious signs of malignancy such as infiltration of the surrounding tissue and increased risk of recurrence after total resection (1).

Cytogenetic and molecular genetic studies have revealed that alterations of chromosome 22 can frequently be observed in meningioma (2, 3). In particular, the region around the *NF2* locus at chromosome 22q12 often shows a loss of heterozygosity in combination with mutations at the second allele in about 60–70% of all meningiomas, thus suggesting a crucial role for this gene in meningioma (4–7). However, there is also a considerable percentage of meningiomas without detectable alterations in this region, suggesting that additional genes may be involved in the development of the tumors (8).

Different studies have indicated that meningiomas are capable of inducing an immune response in the host (9–11). Data from recent studies indicate that some tumors elicit an immune response through the expression of specific antigens, such as 14C1 and HER-2/neu (12–15). Tumor-associated antigens recognized by the immune system may indicate intracellular changes. Until now, most of these tumor-associated antigens have been described for malignant tumors, but we have recently identified several such antigens for meningioma as well (14, 15).

Here, we report the finding of some new tankyrase-related cDNA clones that have been isolated with sera from patients suffering from meningiomas. We performed immunoscreening experiments of a human fb cDNA library using three sera pools from patients bearing tumors corresponding to the three WHO meningioma grades. We identified a considerable number of cDNA clones with homologies to tankyrase. Additionally, 6 of 13 of the common-type meningioma sera tested could detect these clones. However, only 1 of 24 normal sera could detect the clones. Finally, we tried to correlate the immune response to the tankyrase-related clone with expression of the gene in the different meningiomas. Northern blot results revealed expression for two common-type meningiomas. The sera from these patients also reacted positively in the immunoscreening. RT-PCR[3] revealed further expression in normal tissue and in an anaplastic meningioma that was negative in the immunoscreening. Thus, we cannot conclude that the immune response against the tankyrase-related clone is correlated to its

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[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; poly(A)$^+$ mRNA, polyadenylated mRNA; fb, fetal brain.

expression. Nevertheless, we suggest a possible involvement of the tankyrase-related protein in the development of meningioma because we could demonstrate that sera from patients with common-type meningiomas are more reactive against this clone than sera from patients bearing meningiomas of higher grades and from healthy controls.

MATERIALS AND METHODS

cDNA Expression Library Construction. Human poly(A)$^+$ mRNA was used to construct the cDNA expression library in the ZAP Express vector arms (Stratagene) of lambda phage as described previously (13, 15). The human poly(A)$^+$ mRNA is commercially available from Clontech (catalogue number 6525-1).

Immunoscreening of Recombinant Proteins. Blood serum from meningioma patients was isolated from 10-ml samples using serum gel monovettes and stored at −75°C. Before use in immunoscreening, the serum was preabsorbed five times against *Escherichia coli* XL1 Blue MRF′ cells and also preabsorbed five times against bacteria lysed by nonrecombinant ZAP Express phages as described previously (14, 15). The preabsorbed serum was diluted to a final concentration of 1:100 in 1× Tris-buffered saline, 0.5%(w/v) dry milk, and 0.01% thimerosal. We generated three sera pools, each of which consisted of four sera, mainly from one meningioma grade. Pool I consisted of sera from patients with meningioma of the arachnothelial type, meningioma of the fibroplastic type, regressive changed meningioma grade I, and one meningioma that was later classified as syncytial meningioma of the atypical type. Pool II contained sera from three patients with meningioma of the fibroblastic type and one patient with meningioma of the microcystic type. Sera-Pool III consisted of sera from one patient with a recurrent anaplastic meningioma who formerly had an atypical meningioma and one patient with a clearly anaplastic meningioma. One serum was obtained from a patient who formerly had an anaplastic meningioma, and one serum was later classified as syncitial meningioma of the atypical type. We obtained anonymous blood samples from persons without obvious disease as normal sera control.

E. coli XL1 Blue MRF′ cells were transfected with the fb cDNA expression library and plated to an approximate density of 10,000 plaque-forming units/plate as described previously (14, 15). Recombinant protein expression was induced by applying Duralose UV membranes (Stratagene) soaked in 10 mM isopropyl-1-thio-β-D-galactopyranoside. The membranes were blocked with 5%(w/v) dry milk before incubation for 3.5–4 h with diluted serum as described previously (13, 15). Antigen-antibody complexes were detected with an alkaline phosphatase-conjugated goat antihuman IgG antibody, followed by incubation with 0.005% (w/v) 5-bromo-4-chloro-3-indolyl phosphate prediluted in 100% (w/v) dimethylformamid and 0.01% (w/v) nitroblue tetrazolium prediluted in 70% (w/v) dimethylformamid in 1× color developing solution as described previously (14, 15).

Characterization of Positive Clones. Serum-positive phage clones were isolated, and the pBK-CMV phagemids were excised *in vivo* using the ExAssist Interference-Resistant Helper Phage following the manufacturer's instructions (Stratagene). The isolated phagemids were propagated in *E. coli* XLOLR cells, and phagemid DNA was isolated using the QIAprep spin miniprep kit following the manufacturer's instructions (Qiagen). The length of the DNA inserts cloned into the pBK-CMV phagemids was determined by performing a double *Eco*RI and *Xho*I restriction endonuclease digestion following the manufacturer's instructions (Boehringer Mannheim). Standard Tris-acetate/EDTA electrophoresis buffer agarose gel electrophoresis was used to size fractionate the DNA restriction fragments and appropriate standard DNA molecular weight markers (Boehringer Mannheim).

Sequencing and Alignments. Sequencing was performed using the Perkin-Elmer ABIPrism Cycle Sequencing kit according to the manufacturer's instructions. Clone inserts were sequenced with an automated sequencer (LI-COR DNA sequencer model 4000 L). Primers used for the sequencing reaction were the M13 universal primer (5′-TGTAAAACGACGGCCAGT-3′) and the M13 reverse primer (5′-CAGGAAACAGCTATGACC-3′). Sequence alignments with sequences in the European Molecular Biology Laboratory and GenBank databases were performed using the BLASTN, BLASTX, and ClustalW algorithms. Protein structure prediction was performed using the Simple Modular Architecture Research Tool algorithm, TMHMM,[4] and the DAS[5]-transmembrane prediction server.

Northern Blot Analysis. Isolation of RNA from frozen tissue samples was performed with the Trizol kit (Life Technologies, Inc.) according to the manufacturer's instructions. Twenty μg of each RNA were separated in a formaldehyde-agarose gel and transferred to a nylon membrane (GeneScreen; NEN Life Science Products, catalogue number NEF983). The membrane was hybridized overnight with a radioactively labeled DNA probe. The probe was PCR derived with the following primer pair: NotankF2 (5′-AGGAGGCCTTGTACCTTTGC-3′) and NotankR2 (5′-AATGTCTGCCTTGGCTAT).

RT-PCR Analysis. RNA from frozen tissue samples was reverse-transcribed into cDNA using the Omniscript Reverse Transcription Kit (Qiagen, catalogue number 205110) according to the manufacturer's instructions. The concentrations of the resulting cDNA were measured spectrophotometrically. Solutions of the same concentration were generated and used for PCR. We used the Notank F2/R2 primer pair mentioned above for the PCR. An additional PCR reaction using the same cDNAs but with β-actin primers was performed as a control. The primers used had the following sequences: (*a*) ACTB forward, 5′-ACTCTTCCAGCCTTCCTTCC-3′; and (*b*) ACTB reverse, 5′-ACTCGTCATACTCCTGCTTGC-3′.

RESULTS

To investigate whether antigens specific to the three WHO meningioma grades could be identified, we performed immunoscreening following the serological identification of antigens by recombinant expression cloning immunological screening method as described previously (14–16), using a human fb cDNA library with three sera pools from patients bearing meningiomas of differ-

[4] http://genome.cbs.dtu.dk/services/TMHMM.
[5] http://www.sbc.su.se/~miklos/DAS.

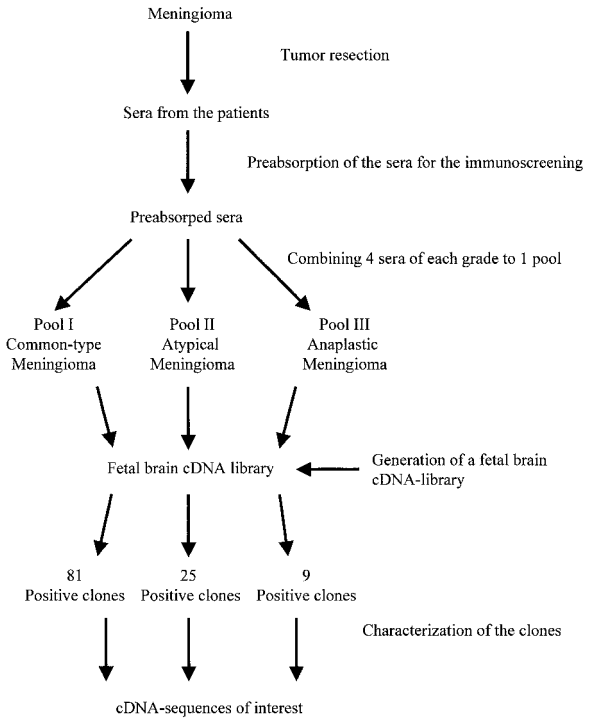

Fig. 1 Overview of the immunoscreening.

ent grades (Fig. 1). The use of a fb cDNA library should provide a highly diverse pool of DNA sequences encoding proteins that may be detected with antibodies in the sera. The cDNA library was generated by reverse transcription of human fb poly(A)$^+$ mRNA and cloning cDNA fragments larger than 500 bp into the ZapExpress vector. After packaging and amplification, the library had a titer of 6×10^9 plaque-forming units/ml. The complexity of the library was tested by using PCR to rescue five genes, *GAPDH*, a SWAP 70 homologous gene, and three glioma-associated genes (*GAS16, GAS64*, and *GAS89*). Recombinant proteins encoded by the sequences of the human fb cDNA library were expressed in *E. coli* XL1 Blue MRF′ cells.

The immunoscreening was performed with pools of sera from different patients bearing the three WHO grades of meningioma. Pool I consisted predominantly of sera from patients with common-type meningiomas, pool II consisted predominantly of sera from patients with atypical meningiomas, and pool III consisted predominantly of sera from patients with anaplastic meningiomas. Histological classification was performed by the Department of Neuropathology according to the WHO classification. All single sera had been preabsorbed as described previously (14, 15). Using the pools of sera from different patients potentially provides a large number of different antibodies for each of the three meningioma grades. We screened 300,000 cDNA clones with each of the sera pools. We detected 126 cDNA clones (fb1 clones) from pool I, 37 cDNA clones (fb2 clones) from pool II, and 25 cDNA clones (fb3 clones) from pool III (Fig. 2). The detected clones were isolated and subjected to a secondary screening to confirm the signals obtained in the primary screening (Fig. 2). After the secondary screening, 81 of the 126 fb1 clones detected from pool I sera remained positive, 25 of the 37 fb2 clones detected from pool II sera remained positive, and 9 of the 25 fb3 clones detected from pool III sera remained positive. The positive phage clones were excised *in vivo*, and the phagemids were propagated in *E. coli* XLOLR cells.

The cloned cDNA inserts were isolated and partially sequenced from both sides, about 1000 bp from the 5′- and 3′-end of the clones. We have obtained partial sequences for 79 of the 81 fb1 clones. Among these clones, there were 16 overlapping cDNA sequences. Nineteen of the fb2 clones have been partially sequenced. In addition to the 16 fb1 clones, we identified two cDNA sequences among these fb2 clones that were homologous to the 16 fb1 clones. All of the sequences overlapped and generated a sequence of 4349 bp (Acc. no. AF305081), in which an open reading frame of 3800 bp was identified. The first ATG start codon can be found at position 308, although the bases in front of this ATG are also in the same open reading frame. The open reading frame ends at position 3800 with a TGA stop codon. The open reading frame obtained demonstrates high homologies along the entire length to cDNA sequences similar to ankyrin and cDNA sequences similar to the telomeric repeat binding factor-1 interacting ankyrin-related ADP-ribose polymerase tankyrase (BLASTN dbest). On the protein level, our sequence also showed high homologies to tankyrase. In both proteins, 77% of the amino acids are identical and 84% have the same functions, indicating that we may have discovered a tankyrase-related protein. The search for known protein domains and comparison with other proteins also revealed homologies to tankyrase. A direct comparison of both sequences using the ClustalW algorithm by the European Molecular Biology Laboratory shows that both proteins are very homologous to each other (Fig. 3). The SMART program, which allows an architectural prediction of proteins, reveals a similar domain structure for both proteins (Fig. 4). With regard to the ankyrin repeats, the sterile alpha motif domain, and the poly(ADP-ribose) polymerase domain, both proteins are very similar. In addition, they contain some transmembrane regions in different positions. According to the SMART program, both proteins contain a transmembrane domain at the NH_2 terminus, and the known tankyrase contains one more transmembrane domain at its COOH-terminal end (Fig. 3).

To further investigate whether the positive signals we obtained using the sera pools resulted from only one serum or from more than one serum, we tested the four single sera belonging to pool I with one of the isolated tankyrase-related clones. With three of these sera, clearly positive signals were obtained, demonstrating that the signals did not result from one highly reactive serum alone. Nine additional common-type sera were tested, and three were reactive. In total, 6 of 13 single sera tested were reactive to the isolated tankyrase-related clone, or 37.5% of the common-type sera tested were positive.

In addition to the tankyrase-related clones, we also detected several clones with very high homologies to ankyrin. When we screened the 13 common-type sera mentioned above, we found 2 of them to be reactive with these ankyrin clones. Of these two reactive sera, only one serum also detected the tankyrase-related clone at the same time. Except for this serum, the other reactive sera only detected either the tankyrase-related clone or ankyrin. Despite the high homologies between our tankyrase-related

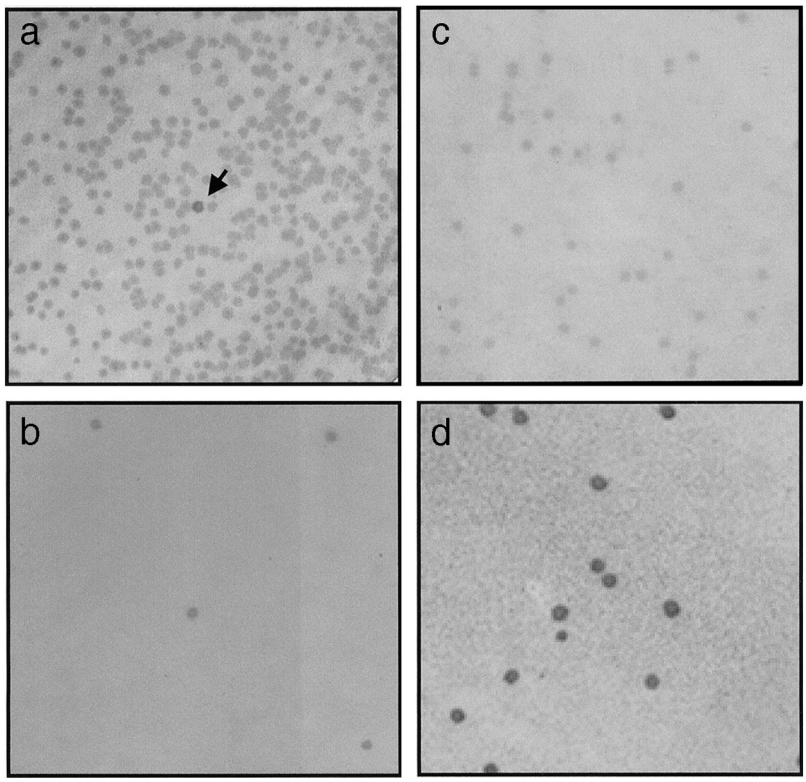

Fig. 2 Screening of a fb expression library with a sera pool containing four sera from patients with common-type meningioma (*a*). The *arrowhead* in *a* indicates the positive clone fb1–1 on the filter of the primary screening. Positive clones from the primary screening were isolated, amplified, and subjected to a secondary screening to test their specificity, as shown for clone fb1–1 (*b*). The isolated tankyrase-related clones were also screened with normal sera pools, as shown for clone fb1–1 (*c*). A clone formerly isolated with the corresponding normal serum served as a positive control (*d*).

clone and tankyrase, we could not detect tankyrase with any of our sera.

Four of the isolated fb1 clones spanning the whole length of our sequence were subjected to an additional immunoscreening with six sera pools consisting of four sera each from people without obvious disease ("normal" sera). Isolated normal antigen clones identified from previous immunoscreening experiments and reactive with corresponding normal sera pools were used as positive controls in these experiments. Only one of the normal sera pools reacted with the tankyrase-related clones, indicating that at least one of the sera in this pool contained antibodies against the corresponding proteins. After testing the four single sera of this pool, only one of the sera was reactive with the tankyrase-related clones, which means that 1 of 24 or 4.2% of the normal sera we tested were reactive.

To investigate whether the immune response against the tankyrase-related clone shown with the immunoscreening experiments might be correlated with its expression, we performed a Northern blot analysis including tumor RNA that was available from patients whose sera had been used for the immunoscreening. We used three RNA samples from common-type meningioma, one RNA sample from an atypical meningioma, and one RNA sample from an anaplastic meningioma for the Northern blot analysis. As shown in Fig. 5, we obtained a signal of 4.3 kb, which is consistent with the predicted length of 4349 bp of the tankyrase-related clone. Sera from both patients bearing these tumors had also been reactive to the expressed tankyrase-related protein in the immunoscreening experiment. The third common-type meningioma RNA used for the Northern blot did not generate a signal. The corresponding serum had also been negative in the immunoscreening. Additionally, we performed a RT-PCR. Again, we could show expression for the same two patients with common-type meningiomas whose sera had also been reactive in the immunoscreening (Fig. 6). Moreover, we also obtained signals with an anaplastic meningioma that was negative in the immunoscreening and with three of four normal tissues used.

DISCUSSION

Studies of different tumors indicate that the expression of immunogenic antigens commonly appears in many neoplasms. Whereas the first investigations were restricted to the analysis of malignant tumors, the isolation of immunogenic antigens in meningiomas has been reported recently (14, 15). In this study, we report the isolation of a new tankyrase-related protein with sera from patients suffering from meningioma.

In addition to several other clones, we detected 18 cDNA clones with homologies to tankyrase (17). Sixteen of these clones were isolated with a sera pool predominantly containing the sera from patients with common-type meningioma. The two remaining clones were found with the sera from patients with atypical meningioma, and none were found with the sera from patients with anaplastic meningioma. None of the other isolated cDNA sequences detected in the screening were found with such a high frequency. The DNA sequences of all 18 tankyrase-related cDNA clones overlapped to form one sequence showing high homology to tankyrase on nucleotide as well as amino acid levels. Using several online programs to predict the domain structure of our protein, we detected at least one transmembrane domain in both the known tankyrase and our tankyrase-related

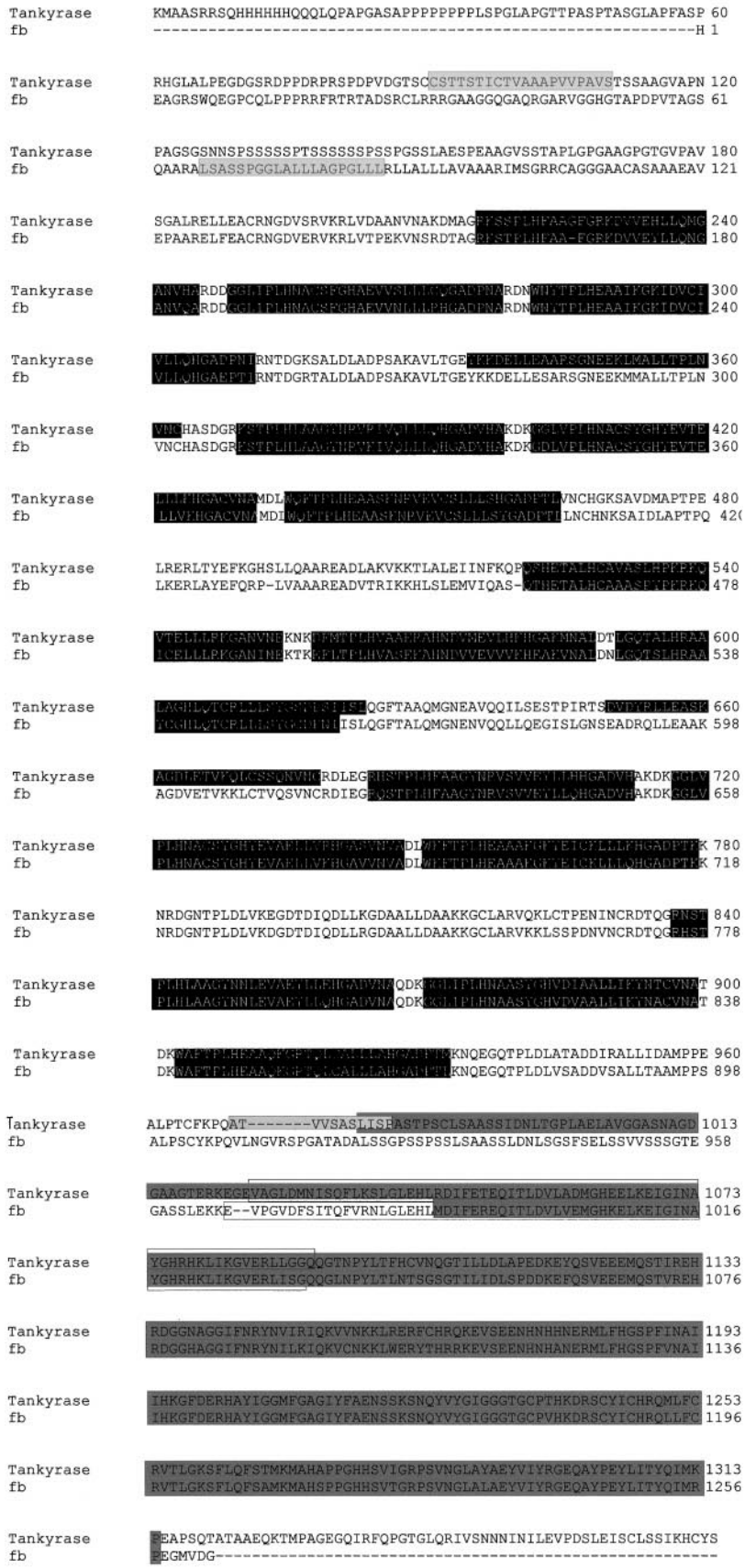

Fig. 3 Alignment (ClustalW) of the known tankyrase and the tankyrase-related protein (fb). The characteristic ankyrin domains are shown in *black boxes*, the SAM domain is shown in a *dark gray box*, and the poly(ADP-ribose) polymerase domain is shown in a *white box*. In addition, the transmembrane domains in each of the proteins are indicated in *light gray boxes*.

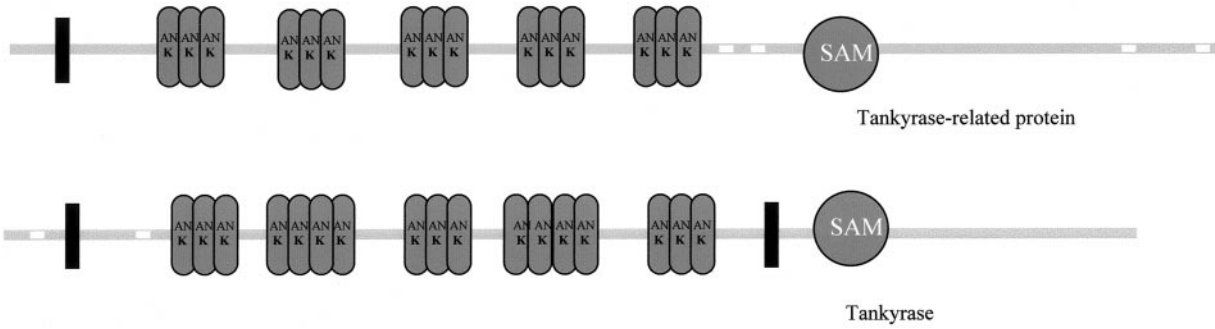

Fig. 4 Comparison of the domains detectable in tankyrase and the tankyrase-related protein using the online program SMART. Both proteins possess a sterile α motive SAM and several ankyrin domains. Additionally, the program detected some transmembrane domains (*black bars*) and some possible transmembrane domains (*white bars*).

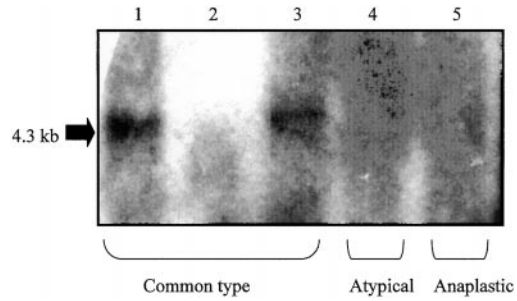

Fig. 5 Northern blot with different meningiomas. *Lanes 1–3*, common-type meningiomas; *Lane 4*, atypical meningioma; *Lane 5*, anaplastic meningioma. The *arrowhead* indicates the positive signals at 4.3 kb.

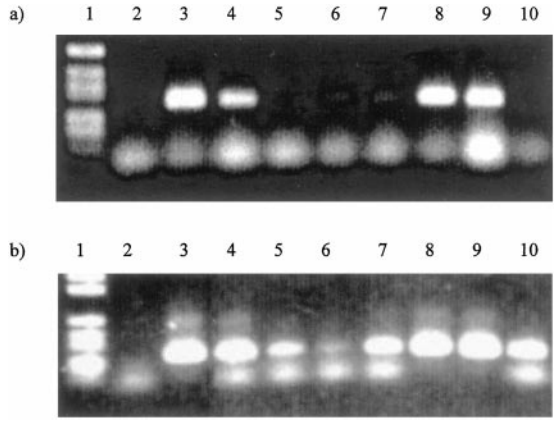

Fig. 6 *a*, RT-PCR with tankyrase-related clone-specific primers using cDNAs from different meningiomas and normal tissues. *Lane 1*, DNA molecular weight marker VI (Roche); *Lane 2*, negative control; *Lanes 3* and *4*, common-type meningiomas; *Lane 5*, atypical meningioma; *Lane 6*, anaplastic meningioma; *Lane 7*, muscle; *Lane 8*, stomach; *Lane 9*, spleen; *Lane 10*, blood. *b*, RT-PCR with β-actin-specific primers using the cDNAs from the same meningiomas and normal tissues as described in *a*.

protein, indicating that they may also be anchored at the cell membrane.

The known tankyrase shows high homologies to ankyrins, proteins that intermediate between transmembrane proteins and proteins belonging to the cytoskeleton. Ankyrins serve as signal transducers between the two groups of proteins and thus serve as signal transducers between the extracellular space of the cell and its inside. Both tankyrase and ankyrins contain a known number of ankyrin repeats that are commonly found in many proteins (17). To date, about 400 proteins of different families and function have been found to contain ankyrin repeats (18). Ankyrins and the tankyrase, however, contain the same number of ankyrin repeats (24 repeats), suggesting a relation between both proteins. Although ankyrins are associated with the cell membrane, the known tankyrase is located in the nucleus and has recently been identified as being located at human telomeres, where it is involved in the regulation of telomere length maintenance (17).

Thus, the tankyrase-related protein may be associated with the cell membrane, where it could be involved in the interactions between the extracellular matrix and the cytoplasm of cells. In this case, the tankyrase-related protein would show a functional similarity to the NF2 gene product that connects transmembrane proteins to components of the cytoskeleton in the same position as the ankyrins. We already have some evidence that in meningioma, the communication between the extracellular matrix and the inside of the cells may be altered. For example, we have identified a novel hyaluronidase expressed by meningiomas (14). The substrate for hyaluronidase is hyaluronan, a component of the extracellular matrix. The protein expressed by the newly isolated tankyrase-related clone may also be involved in the interaction between the cytoskeleton and the outside of cells.

As alluded to previously, we detected the tankyrase-related clones with different frequencies when using the three sera pools. Interestingly, we detected fewer or no tankyrase-related clones when using sera from patients with grades II and III meningioma, respectively. This may be consistent with previous studies showing that in patients with malignant tumors, the immune response is often weakened (19). It may also be interesting that the tankyrase-related clones were detected much more often in our screening than all other cDNA clones that we identified. Additionally, screening the tankyrase-related clones with 13 single common-type sera including all sera from pool I revealed that 6 sera (37.5%) were reactive. However, when screening the cDNA clones with normal sera pools, we only detected the tankyrase-related clones with one of six pools and

with one of the four single sera of this positive pool. This corresponds to only 4.2% reactivity, showing a noteworthy difference to the reactivity found with the common-type sera.

Northern blot experiments indicated that the immune response demonstrated by the immunoscreening was correlated with RNA expression of the tankyrase-related clone because we could demonstrate expression in two common-type meningiomas from patients whose sera also detected the tankyrase-related clone in the immunoscreening. The results of the RT-PCR, however, revealed additional expression in the anaplastic meningioma and in three normal tissues. From these results, the immune response against the tankyrase-related clone does not seem to be clearly correlated with its expression. Nevertheless, this is not very surprising because major tumor antigens are classified into four main categories: (*a*) those with tumor-specific expression; (*b*) those that result from point mutations; (*c*) those encoded by overexpressed genes; and (*d*) those encoded by differentiation antigens (20). To date, we cannot be certain which of these reasons may be responsible for the immunogenicity in the case of meningioma.

Because our tankyrase-related clone shows high homologies to ankyrin as well as to the known tankyrase, the question remains: were the antibodies that detected the tankyrase-related clone specific to this protein, or was the immune response influenced by cross-reactivity? In the primary screening, we also had isolated several clones with high homologies to ankyrin. Screening them with the 13 single common-type sera that we had tested for reactivity against the tankyrase-related clone, we found two sera that were reactive to ankyrin as well. Only one of these two sera belonged to the group of the six sera that were reactive to the tankyrase-related clone. Thus, there are at least five common-type sera that only detect the tankyrase-related clone. In addition, we never detected the known tankyrase with any of the sera used in our screening. We therefore conclude that there are specific antibodies against the tankyrase-related clone that are capable of recognizing the particular protein.

In previous studies, our group has already identified seven novel immunogenic antigens expressed in meningioma, meningioma expressed antigens (14, 15). These MGEAs had been identified using a cDNA library established from a meningioma specimen and the autologous patient serum. Relying on these data, we wanted to find more immunogenic antigens in meningioma. Whereas in the previous study, the aim was to find immunogenic antigens in meningiomas, we tried to find differences in the antigen variety among the three meningioma grades. We also focused on the different frequencies with which single clones had been identified. That is why the tankyrase-related clone, which had been isolated the most often, seemed worthwhile for further investigation. We were not able to detect any of the previously described MGEAs in our present study. However, because we used a different cDNA library and different sera, this discrepancy may not be too surprising. It has already been indicated previously that the seven MGEAs may represent only part of a wider spectrum of antigens expressed in meningiomas (15). With the novel sequences that we identified with the sera pools of different meningioma grades, we could demonstrate again that benign tumors such as meningiomas express immunogenic antigens.

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