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(54) **SOMATIC MUTATIONS IN ATRX IN BRAIN CANCER**

SOMATISCHE ATRX-MUTATIONEN BEI HIRNKREBS

MUTATIONS SOMATIQUES DE L'ATRX DANS LE CANCER DU CERVEAU

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Description

[0001] This invention was made using funds from the U.S. governments. The U.S. retains certain rights to the invention under the terms of National Institutes of Health grants CA121113, P50CA062924, P01CA134292, R01CA113669, RO1CA 43460 and CA57345, CA1403160, 5P50-N5020023-28 (SRC5R37), and CA011898-41.

TECHNICAL FIELD OF THE INVENTION

[0002] This invention is related to the area of cancer. In particular, it relates to brain cancers.

BACKGROUND OF THE INVENTION

[0003] Telomeric DNA functions to stabilize chromosomal ends and is progressively lost during cell division (the end replication problem), thus limiting cellular proliferative capacity.(1-3) The majority of cancers solve the end replication problem by expressing the telomere-synthesizing enzyme telomerase. A subset of the others utilizes a genetic recombination-based telomerase-independent telomere maintenance mechanism termed alternative lengthening of telomeres (ALT).(4-7) The prevalence of ALT varies widely, but is found more often in cancers of the central nervous system (CNS) and of mesenchymal tissues than in common epithelial tumors.(8, 9)

[0004] A recent study of pancreatic neuroendocrine tumors (PanNETs) revealed that 43% harbored inactivating mutations in the *ATRX* or *DAXX* genes.(10) Notably, these mutations were mutually exclusive, indicating that they functioned in the same pathway. This mutual exclusivity was intriguing, as independent studies had shown that the proteins encoded by *ATRX* and *DAXX* interact with one another.(11) The *ATRX* gene encodes a large protein possessing a C-terminal helicase/ATPase domain placing it in the SNF2 family of chromatin remodeling enzymes.(12) Inherited mutations in *ATRX* cause X-linked alpha thalassemia/mental retardation syndrome, characterized by multiple developmental abnormalities in affected males.(13, 14) *DAXX* is a nuclear protein that interacts with numerous SUMO-modified proteins and plays a role in transcriptional repression.(15) The *ATRX* and *DAXX* protein complex has been suggested to play multiple cellular roles, including functioning in chromatin remodeling.(11, 16) Notably, the *ATRX/DAXX* complex was recently found to be required for the incorporation of the histone variant H3.3 at telomeres.(17-19) This histone chaperone activity may play a role in establishing or maintaining telomere stability, at least in mouse embryonic stem cells.(20, 21) Reducing either *ATRX* or H3.3 levels in these cells decreased the amount of heterochromatic protein HP1-alpha at telomeres and increased markers of telomere dysfunction.(20, 21) Epigenetic changes in telomeric and subtelomeric chromatin have also been shown to affect telomere length, as well as recombination at telomeres.(22-24)

[0005] There is a continuing need in the art to identify markers for diagnosis, prognosis, stratifying, and targeting of brain tumors.

SUMMARY OF THE INVENTION

[0006] According to one aspect of the invention a method predicts outcome of a central nervous system (CNS) tumor in a patient. The CNS tumor is tested, or cells or nucleic acids shed from the tumor are tested, for the presence of an inactivating mutation in *ATRX*. The mutation is a positive prognostic indicator.

[0007] According to another aspect of the invention a method identifies a CNS tumor. The presence of an inactivating mutation in *ATRX* is tested for and identified in a tissue suspected of being a CNS tumor, or in cells or nucleic acids shed from the tumor. The presence of an inactivating mutation indicates a CNS tumor.

[0008] According to still another aspect, a method inhibits *ATRX* in a CNS tumor. An *ATRX* inhibitory agent is administered to the CNS tumor. The agent inhibits *ATRX* function or expression.

[0009] These and other embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with methods for assessing, identifying, diagnosing, prognosticating, stratifying, and treating tumors of the central nervous system.

BRIEF DESCRIPTION OF THE DRAWINGS**[0010]**

Fig. 1A-1G. Representative images of ALT-negative and ALT-positive tumors. (Fig. 1A) ALT-negative PanNET. Telomere FISH signals are markedly dimmer in PanNET cells (*) than in the surrounding stromal cells (arrowheads). Centromere-specific FISH probe serves as positive control for hybridization. (Fig. 1B and Fig. 1C) Immunolabeling of same PanNET as in A, shows nuclear positivity for *ATRX* and *DAXX* proteins, respectively. (Fig. 1D) Example of ALT-positive PanNET. Large, ultra-bright telomere FISH signals indicative of ALT are indicated (arrows). (Fig.

1E) Immunolabeling of same PanNET as in D, shows loss of nuclear DAXX protein in tumor cells. Benign endothelial cells (arrowheads) serve as positive staining controls. (Fig. 1F and Fig. 1G) Examples of ALT-positive GBM and medulloblastoma, respectively. Original magnification=400X for all images.

5 Fig. 2A-2G. Telomere-FISH and immunofluorescence co-staining in ALT-positive tumors. (Fig. 2A) ALT-positive PanNET telomere FISH and ATRX protein. (Fig. 2B) same image as in A, omitting telomere and DAPI channels, highlighting loss of nuclear ATRX. Benign stromal cells positive for nuclear ATRX protein are indicated by arrows. (Fig. 2C) ALT-positive PanNET co-stained with telomere FISH and DAXX protein. (Fig. 2D) same image as in Fig. 2C, omitting telomere and DAPI channels. Punctate nuclear DAXX staining in benign stromal cells is indicated by arrow heads. E, ALT-positive medulloblastoma stained for telomere FISH and PML protein. Arrows show co-localization of PML protein and ALT-associated telomere foci. F&G, high magnification images of telomere and PML protein co-staining showing typical targetoid appearance of ALT-associated PLM bodies (APB). Original magnification=400X for images Fig. 2A- Fig. 2AE, 1000X for images Fig. 2F and Fig. 2G.

15 **Fig. 3A-3B (S1).** Telomere-FISH and immunofluorescence co-staining for ATRX protein. (**Fig. 3A**) ALT-positive osteosarcoma cell line U2-OS showing lack of nuclear ATRX protein. (**Fig. 3B**) ALT-negative PanNET cell line BON-1 showing ATRX nuclear-positivity. In both cases the nuclear DNA was counter stained with DAPI. Original magnification=400X.

20 DETAILED DESCRIPTION OF THE INVENTION

[0011] The inventors have found that ATRX mutations are frequently found in tumors of the central nervous system. Moreover, they are mutated together with IDH1 or IDH2 and TP53. ATRX mutations appear to be a later event in the progression of the brain tumors than the other mutations. Thus somatic mutations in ATRX can be used as a diagnostic, prognostic, or stratifying factor for such tumors.

[0012] Tumors of the CNS which may be assessed and treated include without limitation medulloblastoma, oligodendroglioma, pediatric glioblastoma multiforme, adult glioblastoma multiforme, oligoglioma, anaplastic oligodendroglioma, oligoastroglioma, anaplastic oligoastrocytoma, astrocytoma, anaplastic astrocytoma, ependymoma, anaplastic ependymoma, myxopapillary ependymoma, subependymoma, mixed gliomas, polar spongioblastomas, astroblastoma, gliomatosis cerebri, medulloepithelioma, neuroblastoma, retinoblastoma, and ependymoblastoma. Glial tumors of any type may be assessed and treated.

[0013] Tests for ATRX mutations can be performed using protein based or nucleic based assays. Sequence determination of the nucleic acid can be used to identify mutations. Probes or primers, and kits and techniques employing both can be used. PCR or other specific or global amplification can be used. Mutations can be identified in any available genetic material including or example genomic DNA, cDNA, and RNA. Nucleic acids can be amplified, enriched, and/or purified prior to assessment Protein based assays may involve specific antibodies and/or ATRX binding partner DAXX. The antibodies may be polyclonal or monoclonal, fragments (Fab, Fab'), single chain constructs (scFv), etc. Nucleic acid based assays include without limitation, hybridization to probes, amplification using specific primers, primer extension, ligation assay, etc. Any of these techniques can also be combined. Assays can be performed together with tests for other gene mutations or alterations of the genome. Results can be integrated and used to accurately and comprehensively characterize and/or identify a tumor or the patient.

[0014] Results of assays can be recorded in a written medium, an electronic medium, or transmitted orally or electronically to a health care provider, a patient, a family member, a hospital, etc. Testing requires physical steps, and typically involves chemical changes to occur to a test sample. Typically the test sample is a sample that is removed from the patient body, so that the test is performed outside of a patient body.

[0015] Samples which may be tested include without limitation brain tissue, tumor tissue, CNS fluid, neuronal tissue, blood, urine, saliva, tears, sputum, etc. These samples may be collected and processed and/or stored prior to testing. The samples may be frozen or fixed. They may be archival or freshly collected. Typically the tissue or body fluid will be isolated from the body and the assay will be performed *ex vivo* on the isolated sample.

[0016] ATRX inhibitory agents as used in this specification inhibit either ATRX function or expression. Such agents may be an antibody, an antibody fragment, or a single chain antibody construct Alternatively it can be an inhibitory RNA or other inhibitory nucleic acid molecule, including but not limited to antisense oligonucleotides, antisense expression constructs, siRNA, and RNAi.

[0017] Any type of mutation may be identified. Inactivating mutations include without limitation R2079X, Q1874X, Q1788H, E2277K, Q2156H, K455X, W263X, R2153C, and R1803H. The mutation may be, for example, a frameshift mutation, a splice-site mutation, an indel (insertion or deletion) mutation, a large genomic rearrangement, or a missense mutation. Typically an indel may involve a small portion of a gene, such as 1-10 nt. A large rearrangement may involve large portions or all of a gene, such as greater than 10%, greater than 25%, greater than 50%, greater than 75% or

greater than 100% of a gene. Particular mutations which may be identified include g.chrX:76778161_76778162insA; g.chrX:76824745_76824748delTCTC; g.chrX:76741670_76741673delCTAT; g.chrX:76798738_76798741delACTA; g.chrX:76665385C>A; g.chrX:76825188_76825194delTTGAGGA; g.chrX:76831065delG(hom); g.chrX:76806828_76806829insT; g.chrX:76825743_76825744delTG; g.chrX:76798774_76798775delAG(hom); g.chrX:76826615C>T(hom); g.chrX:76700843G>A(hom); and g.chrX:76760970C>T.

[0018] Stratification of patients can be used to assign a treatment regimen. It may be used in prospective or retrospective clinical studies. It can be used to assign a prognosis. Stratification typically assigns a patient to a group based on a shared mutation pattern or other observed characteristic or set of characteristics.

[0019] The above disclosure generally describes the present invention. All references disclosed herein are expressly incorporated by reference. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1-- ATRX and DAXX gene mutations correlate with ALT-positivity

[0020] Given the potential role of ATRX and DAXX in modulating telomeric chromatin, we evaluated telomere status in pancreatic neuroendocrine tumors (PanNETs) with known ATRX and DAXX mutational status. Telomere-specific fluorescence in situ hybridization (FISH) was used to directly assess the telomeres in PanNETs. Neoplasms with ALT are readily distinguishable by large ultra-bright telomere FISH signals - a nearly universal feature of ALT-positive cell populations. (25) Although telomere FISH signals from these individual bright foci have often been shown to colocalize with PML protein, this localization is not as reliable as the strength of the FISH signals and was not used for classification in our study.(26-28)

[0021] Twenty-five of the 41 PanNETs (61%) examined by telomere FISH displayed evidence of ALT (Table 1, Figure 1). Importantly, ALT was not observed in any of the surrounding non-neoplastic cells, including stromal fibroblasts, pancreatic acini, pancreatic ducts and islets of Langerhans (Figures 1&2).(29) ATRX and DAXX gene mutations both were significantly correlated with ALT-positivity (p<0.008 for either gene). In particular, all 21 (100%) PanNETs with ATRX or DAXX gene mutations were ALT-positive by telomere FISH (Table 1).

Table 1. PanNET cases grouped according to mutation status for ATRX and DAXX and displaying ALT-status and immunohistochemistry of ATRX and DAXX

Gene Status	Case	ALT-status*	ATRX IHC†	DAXX IHC†
ATRX Point mutations & indels	PanNET5	Pos	Neg	Pos
	PanNET13	Pos	Neg	Pos
	PanNET27	Pos	Neg	Pos
	PanNET35	Pos	Neg	Pos
	PanNET52	Pos	Neg	Pos
	PanNET59	Pos	Pos	Pos
	PanNET61	Pos	Neg	Pos
	PanNET78	Pos	Pos	Pos
	PanNET85	Pos	Neg	Pos
	PanNET112‡	Pos	Neg	Pos
DAXX Point mutations & indels	PanNET25	Pos	Pos	Het
	PanNET31	Pos	Pos	Het
	PanNET44	Pos	Pos	Neg
	PanNET56	Pos	Pos	Neg
	PanNET77	Pos	Pos	Neg
	PanNET80	Pos	Pos	Neg
	PanNET84	Pos	Pos	Het
	PanNET87	Pos	Pos	Het
	PanNET93	Pos	Pos	Neg
	PanNET104	Pos	Pos	Neg
PanNET133	Pos	Pos	Neg	
	PanNET6	Neg	Pos	Pos
	PanNET10	Neg	Pos	Pos
	PanNET21	Neg	Pos	Pos

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(continued)

Gene Status	Case	ALT-status*	ATRX IHC†	DAXX IHC†
	PanNET24	Neg	Pos	Pos
	PanNET29	Pos	Pos	<i>Het</i>
	PanNET36	Neg	Pos	Pos
	PanNET39	Pos	Neg	Pos
	PanNET45	Pos	Pos	<i>Neg</i>
	PanNET57	Neg	Pos	Pos
	PanNET63	Neg	Pos	Pos
ATRX/DAXX WT for Point mutations & indels	PanNET64	Pos	Het	Pos
	PanNET66	Neg	Pos	Pos
	PanNET69	Neg	Pos	Pos
	PanNET79	Neg	Pos	Pos
	PanNET83	Neg	Pos	Pos
	PanNET91	Neg	Pos	Pos
	PanNET121	Neg	Pos	Pos
	PanNET126	Neg	Pos	Pos
	PanNET128	Neg	Pos	Pos
	PanNET129	Neg	Pos	Pos

* The intensity of telomere FISH signals was assessed to be either negative or positive for the ALT phenotype.

† Immunohistochemistry was scored as uniformly positive, negative or heterogeneous for nuclear labeling.

‡ Multifocal tumor, featuring ALT and negative nuclear immunolabeling in the majority of the tumor.

ALT, alternative lengthening of telomeres; Het, heterogeneous; IHC, immunohistochemistry; Neg, negative; PanNET, pancreatic neuroendocrine tumor; Pos, positive; WT, wild type.

[0022] Four of the 20 cases without detectable mutations in ATRX or DAXX were ALT-positive. To determine whether ATRX and DAXX expression were also normal in these tumors, serial sections of the same tumors were immunolabeled with antibodies against the ATRX and DAXX proteins. Each of the four tumors demonstrated loss of nuclear expression of either ATRX (2 cases) or DAXX (2 cases) in either the entire tumor, or confluent parts of the tumor (Fig. 1 and 2). Of note, the nuclear expression of the corresponding wild type partner (either DAXX or ATRX, respectively) was retained in each of these four cases. Though the immunohistochemical results on tumors with ATRX or DAXX mutations were expected, they provide excellent controls for the specificities of the antibodies used for immunolabeling. Notably, each of the 16 tumors without ALT showed robust nuclear labeling for both ATRX and DAXX (Table 1). The relationship between ALT-positivity and abnormal immunolabeling for either the ATRX or DAXX proteins was statistically significant ($p=0.012$ and $p=0.003$, respectively).

[0023] In sum, there was a perfect correlation between the absence of nuclear ATRX or DAXX expression and the ALT phenotype and >80% of the 23 tumors without ATRX or DAXX expression could be accounted for by point mutations and small indels, *i.e.*, inactivating mutations of the ATRX or DAXX genes.

EXAMPLE 2-- ATRX mutations in cancers of the CNS

[0024] To ascertain whether ATRX and DAXX gene mutations might be more generally associated with the ALT-phenotype, we examined 447 tumors of other types. We identified a low to moderate frequency of ATRX mutations in cancers of the CNS. Specifically, mutations were detected in 2 of 18 (11.1%) pediatric GBM, 8 of 123 (6.5%) adult GBM, 1 of 13 (7.7%) oligodendrogliomas, and 1 of 65 (1.5%) medulloblastomas. No mutations in either ATRX or DAXX were identified in 11 adult neuroblastomas, or in 25 ovarian adenocarcinomas, 96 breast adenocarcinomas and 96 pancreatic adenocarcinomas. (Table 2).

[0025] Further, ATRX mutations were found in anaplastic astrocytomas (41%), astrocytomas (29%), anaplastic oligoastrocytomas (20%), oligoastrocytomas (33%), anaplastic oligogliomas (7%), and oligogliomas (14%). These mutations were highly associated with mutations in IDH1/2 and TP53.

EXAMPLE 3

[0026] To determine if the ALT status of these additional tumor types also correlated with the presence of somatic

ATRXX mutations, we performed telomere FISH as described above on 8 *ATRXX* mutant cases in which tumor material was available. In each of the eight cases, extremely bright telomeric foci indicating ALT were identified in the neoplastic cells but not in the non-neoplastic cells surrounding them (examples in Fig.1 and 2). Immunolabeling with antibodies to DAXX or ATRX showed that these tumors had lost nuclear expression of ATRX. As controls, we studied 23 *ATRXX* and *DAXX_wild* type tumors of the same types with the identical techniques. None of the tumors without *ATRXX* or *DAXX* mutations demonstrated the ALT phenotype by telomere FISH and all 23 retained robust nuclear labeling for both ATRX and DAXX.

EXAMPLE 4-*ATRXX* mutated in established cancer cell line

[0027] For future mechanistic studies, we considered it important to determine whether a human cancer cell line with a mutation in *ATRXX* or *DAXX* could be identified. We chose to study U-2 OS, derived from an osteosarcoma, as this line was a prototype for delineating the ALT phenotype.(7) We found that exons 2 to 19 of *ATRXX* were homozygously deleted in these cells, unequivocally inactivating the gene product, causing a lack of ATRX immunolabeling in these cells (Suppl. Fig. S1).

EXAMPLE 5-Discussion

[0028] The results described above show a remarkable correlation between inactivation of *ATRXX* or *DAXX* and the ALT phenotype in unrelated tumor types. Though we cannot infer from these data that mutations in *ATRXX* or *DAXX* actually cause this phenotype, prior studies provide a mechanism through which this might occur. It has been proposed that ATRX-DAXX function in replication-independent heterochromatin assembly at specific repetitive G-rich regions, such as the telomeres.(16, 18, 19) In patients with the ATRX syndrome, mutations in the *ATRXX* gene lead to changes in the DNA methylation status at subtelomeric regions which, as described below, may impact telomere stability.(30) Furthermore, experimentally decreasing ATRX or H3.3 in mouse ES cells results in telomere destabilization and an up-regulation of telomere repeat-containing RNA (TERRA).(19-21, 31)

[0029] Current evidence indicates that the ALT telomere maintenance mechanism is dependent upon homologous recombination (HR).(8, 32) Telomeres are composed of long tracts of highly repetitive DNA terminating in single stranded 3' ends, which should provide an ideal substrate for HR; thus, it has been proposed that HR at telomeres is actively suppressed in normal cells.(29) In agreement with this, somatic cell telomeric and proximal subtelomeric DNA feature tightly packed nucleosomes organized in a repressive heterochromatic state enriched for repressive epigenetic chromatin marks, such as DNA methylation and trimethylation of lysine 9 on histone H3 and of lysine 20 on histone H4.(22-24, 33) Mice engineered to contain reduced levels of such repressive epigenetic marks possess abnormally long and heterogeneous telomeres, as well as increased levels of inter-chromatid telomere recombination - all of which are hallmarks of the ALT phenotype.(22, 23) Our results are consistent with a model in which loss of ATRX-DAXX function impairs the heterochromatic state of the telomeres, perhaps due to reduced levels of H3.3 incorporation, leading to telomere destabilization and increased HR at the telomeres; thus, facilitating the development of ALT.

EXAMPLE 6--methods

Tissue Samples and Mutational Analysis

[0030] In order to assess the potential consequences of ATRX and DAXX gene mutations on telomeres, 41 sporadic, nonfunctional PanNETs were chosen from a series of PanNETs whose ATRX and DAXX gene mutational status was previously determined by exomic sequencing.(1) Of the 41 PanNETs examined, ten had ATRX gene mutations, eleven had DAXX gene mutations, and the remaining 20 had wild type ATRX and DAXX genes. Details on the specific gene mutations as well as clinicopathologic variables for this PanNET series are presented in Supplementary Table S2. Clinical information on the patients evaluated in this study was obtained from the Johns Hopkins Hospital in the context of approved IRB protocols. The ATRX and DAXX genes were also sequenced in 65 cases of medulloblastoma, 13 cases of oligodendroglioma, 141 cases of glioblastoma multiforma (18 pediatric GBM and 123 adult GBM), 11 neuroblastomas, 25 ovarian carcinomas, 96 breast carcinomas and 96 pancreatic ductal adenocarcinomas.

Immunohistochemistry

[0031] Immunolabeling for the ATRX and DAXX proteins was performed on formalin-fixed, paraffin embedded sections as previously described.(1) Briefly, heat-induced antigen retrieval was performed in a steamer using citrate buffer (catalog# H-3300, Vector Laboratories) for 30 minutes. Endogenous peroxidase was blocked (catalog# S2003, Dako) and serial sections were then incubated with primary antibody; anti-ATRXX (1:400 dilution; catalog# HPA001906, Sigma-

Aldrich, lot R00473) or anti-DAXX (1:150 dilution; catalog# HPA008736, Sigma-Aldrich, lot A39105) for 1 hour at room temperature. The primary antibodies were detected by 30 minute incubation with HRP-labeled secondary antibody (catalog# PV6119, Leica Microsystems) followed by detection with 3,3'-Diaminobenzidine (Sigma-Aldrich), counterstaining with Harris hematoxylin, rehydration and mounting. Only nuclear labeling of either protein was evaluated. The immunolabeled PanNET slides were assessed and scored by 2 authors (R.H. and A.M.); the immunolabeled CNS tumor slides were assessed by F.R. Internal controls included islets of Langerhans in PanNETs and endothelial cells (including within intra-tumoral vessels) which demonstrated strong nuclear immunolabeling for both ATRX and DAXX.

Telomere-specific FISH and microscopy

[0032] Combined telomere-specific FISH and immunofluorescence labeling for ATRX, DAXX and PML was conducted as previously described.(2, 3) Briefly, deparaffinized slides were hydrated, steamed for 20 minutes in citrate buffer (catalog# H-3300; Vector Laboratories), dehydrated and hybridized with a Cy3-labeled peptide nucleic acid (PNA) probe complementary to the mammalian telomere repeat sequence ([N-terminus to C-terminus] CCCTAACCCTAACCCTAA; SEQ ID NO: 1). As a positive control for hybridization efficiency, a FITC-labeled PNA probe having specificity for human centromeric DNA repeats (ATTCGTTGGAAACGGGA (SEQ ID NO: 2); CENP-B binding sequence) was also included in the hybridization solution.(4) Following post-hybridization washes, the desired primary antibody was applied (anti-ATRX, as described above; anti-DAXX, as described above; anti-PML antibody, 45 minute incubation at 1:100 dilution; catalog# PG-M3, DAKO), followed by application of species-appropriate Alexa 488 fluorescent secondary antibody (Molecular Probes Cat.# A-11034 and A-11001) and nuclear counterstaining with DAPI. Slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA) and appropriate fluorescence excitation/emission filters. Grayscale images were captured for using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored and merged. Quantification from the digital images was conducted using Telometer, a custom software plugin created for the open source image analysis program ImageJ, freely available for download (<http://bui2.win.ad.jhu.edu/telometer/>).

[0033] The FISH and immunolabeled slides were assessed and scored independently by 2 authors (C.H. and A.K.M.). The gold standard for determining the presence of ALT is the demonstration of telomere length maintenance over time in a continually growing cell population lacking any evidence of telomerase activity.(5) This standard, however, cannot be applied to fixed clinical specimens. Previously, it was shown that subsets of cells in ALT-positive cell lines harbor unusually large promyelocytic leukemia (PML) nuclear bodies which contain large amounts of telomeric DNA (ALT-associated PML bodies; APBs).(6) APBs are not observed in normal cells, nor are they observed in ALT-negative cancer cells, and thus APBs serve as specific biomarkers of ALT. APBs can be readily visualized in fixed tissues by combining telomere-specific FISH with fluorescent immunostaining for PML protein.(3) This method has been extensively validated and allows for the straightforward identification of ALT-positive cancers in fixed human tissue specimens.(7) APB-associated telomeric DNA aggregates are unique to ALT-positive cell populations and are significantly larger and brighter than the FISH signals emanating from individual telomeres in the same cell population.

[0034] In our study, PanNETs were classified as ALT-positive if they met the following criteria: (1) the presence of ultra-bright, intra-nuclear foci of telomere FISH signals, with integrated total signal intensities for individual foci being > 10 fold that of the per cell mean integrated signal intensities for all telomeric signals in individual benign stromal cells within the same case (Quantitative data on representative individual telomere aggregates in 10 randomly chosen PanNET cases is presented in Supplementary Table S3); (2) $\geq 1\%$ of neoplastic cells displaying ALT-associated telomeric DNA foci. Tumor samples lacking ALT-associated telomeric foci in which at least 5000 cells were assessed were considered ALT-negative. In all cases, areas exhibiting necrosis were excluded from consideration.

References for Example 6 only.

[0035] The contents of each are expressly incorporated herein.

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[0036] The disclosure of each reference cited is expressly incorporated herein.

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CLAUSES

[0037]

- 45 1. A method for predicting outcome of a Central Nervous System (CNS) tumor in a patient, comprising:
 - testing the CNS tumor, or cells or nucleic acids shed from the tumor, for the presence of an inactivating mutation in *ATRX*, wherein the mutation is a positive prognostic indicator.
- 50 2. The method of clause 1 wherein the step of testing comprises an immunohistochemical analysis.
3. The method of clause 1 wherein the step of testing comprises a nucleic acid analysis.
4. A method of identifying a CNS tumor, comprising:
 - 55 testing for and identifying presence of an inactivating mutation in *ATRX* in a tissue suspected of being a CNS tumor, or in cells or nucleic acids shed from the tumor, wherein the presence of an inactivating mutation indicates a CNS tumor.

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5. A method for classifying a tumor of the Central Nervous System (CNS) of a patient, comprising:
- testing the CNS tumor, or cells or nucleic acids shed from the tumor, for the presence of an inactivating mutation in *ATRX*;
 - assigning the CNS tumor to a class based on the presence of the mutation or the identity of the mutation.
6. The method of clause 5 wherein the patient is assigned to a clinical trial group according to the class.
7. The method of clause 5 wherein a treatment is prescribed based on the class.
8. A method of inhibiting *ATRX* in a CNS tumor, comprising:
- administering an *ATRX* inhibitory agent to the CNS tumor, wherein the agent inhibits *ATRX* function or expression.
9. The method of clause 8 wherein the inhibitory agent is an antibody, an antibody fragment, or a single chain antibody construct.
10. The method of clause 8 wherein the inhibitory agent is an inhibitory RNA molecule.
11. The method of clause 1, 4, 5, or 8 wherein the CNS tumor is a glial tumor.
12. The method of clause 1,4, 5, or 8 wherein the CNS tumor is selected from the group consisting of: pediatric glioblastoma, adult glioblastoma, oligodendroglioma, medulloblastoma, anaplastic oligodendroglioma, oligoastrocytoma, anaplastic oligoastrocytoma, astrocytoma, and anaplastic astrocytoma.
13. The method of clause 1,2,4, or 5 wherein the mutation is selected from the group consisting of R2079X, Q1874X, Q1788H, E2277K, Q2156H, K455X, W263X, R2153C, and R1803H.
14. The method of clause 1,4, or 5 wherein the mutation is a frameshift mutation.
15. The method of clause 1,4, or 5 wherein the mutation is selected from the group consisting of a splice-site mutation or an indel mutation.
16. The method of clause 1, 4, or 5 wherein the mutation is a missense mutation.
17. The method of clause 1, 4, or 5 wherein the mutation is selected from the group consisting of
- g.chrX:76778161_76778162insA;
 - g.chrX:76824745_76824748delTCTC; g.chrX:76741670_76741673delCTAT;
 - g.chrX:76798738_76798741delACTA; g.chrX:76665385C>A;
 - g.chrX:76825188_76825194delTTGAGGA; g.chrX:76831065delG(hom);
 - g.chrX:76806828_76806829insT; g.chrX:76825743_76825744delTG;
 - g.chrX:76798774_76798775delAG(hom); g.chrX:76826615C>T(hom);
 - g.chrX:76700843G>A(hom); and g.chrX:76760970C>T.
18. The method of clause 1,4, or 5 wherein the mutation is a large genomic rearrangement.

SEQUENCE LISTING

[0038]

<110> Yan, Hai
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Kinzler, Kenneth W.
Meeker, Alan
Hruban, Ralph
Papadopoulos, Nickolas
Diaz, Luis
Jiao, Yuchen

<120> SOMATIC MUTATIONS IN *ATRX* IN BRAIN
CANCER

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Claims

- 25
1. A method for predicting outcome of a Central Nervous System (CNS) tumor in a patient, comprising:
testing the CNS tumor, or cells or nucleic acids shed from the tumor, for the presence of an inactivating mutation in *ATRX*, wherein the mutation is a positive prognostic indicator.
30
 2. The method of claim 1 wherein the step of testing comprises an immunohistochemical analysis.
 3. The method of claim 1 wherein the step of testing comprises a nucleic acid analysis.
 - 35 4. A method of identifying a CNS tumor, comprising:
testing for and identifying presence of an inactivating mutation in *ATRX* in a tissue suspected of being a CNS tumor, or in cells or nucleic acids shed from the tumor, wherein the presence of an inactivating mutation indicates a CNS tumor.
40
 5. A method for classifying a tumor of the Central Nervous System (CNS) of a patient, comprising:
testing the CNS tumor, or cells or nucleic acids shed from the tumor, for the presence of an inactivating mutation in *ATRX*;
45 assigning the CNS tumor to a class based on the presence of the mutation or the identity of the mutation.
 6. The method of claim 5 wherein the patient is assigned to a clinical trial group according to the class.
 7. The method of claim 5 wherein a treatment is prescribed based on the class.
 - 50 8. An *ATRX* inhibitory agent for use in inhibiting *ATRX* in a CNS tumor, wherein the agent inhibits *ATRX* function or expression.
 9. The *ATRX* inhibitory agent for use according to claim 8 wherein the inhibitory agent is an antibody, an antibody fragment, a single chain antibody construct, or an inhibitory RNA molecule.
55
 10. The method of claim 1, 4 or 5, or the *ATRX* inhibitory agent for use according to claim 8, wherein the CNS tumor is a glial tumor.

- 5
11. The method of claim 1, 4 or 5, or the ATRX inhibitory agent for use according to claim 8, wherein the CNS tumor is selected from the group consisting of: pediatric glioblastoma, adult glioblastoma, oligodendroglioma, medulloblastoma, anaplastic oligodendroglioma, oligoastrocytoma, anaplastic oligoastrocytoma, astrocytoma, and anaplastic astrocytoma.
12. The method of claim 1, 2, 4, or 5 wherein the mutation is selected from the group consisting of R2079X, Q1874X, Q1788H, E2277K, Q2156H, K455X, W263X, R2153C, and R1803H.
- 10
13. The method of claim 1, 4, or 5 wherein the mutation is selected from the group consisting of a frameshift mutation, a missense mutation, a splice-site mutation and an indel mutation.
14. The method of claim 1, 4, or 5 wherein the mutation is a large genomic rearrangement.

15 **Patentansprüche**

1. Verfahren zur Ergebnisvorhersage eines Tumors des zentralen Nervensystems (ZNS) bei einem Patienten, umfassend:

20 Testen des ZNS-Tumors, oder von Zellen oder Nukleinsäuren, die von dem Tumor abgestoßen werden, auf Anwesenheit einer inaktivierenden Mutation in *ATRX*, wobei die Mutation ein positiver prognostischer Indikator ist.

- 25 2. Verfahren nach Anspruch 1, wobei der Testschritt eine immunhistochemische Analyse umfasst.

3. Verfahren nach Anspruch 1, wobei der Testschritt eine Nukleinsäureanalyse umfasst.

4. Verfahren zum Feststellen eines ZNS-Tumors, umfassend:

30 Testen auf und Feststellen von Anwesenheit einer inaktivierenden Mutation in *ATRX* in einem Gewebe, bei dem Verdacht auf einen ZNS-Tumor besteht, oder in Zellen oder Nukleinsäuren, die von dem Tumor abgestoßen werden, wobei die Anwesenheit einer inaktivierenden Mutation einen ZNS-Tumor anzeigt.

- 35 5. Verfahren zum Klassifizieren eines Tumors des zentralen Nervensystems (ZNS) eines Patienten, umfassend:

Testen des ZNS-Tumors, oder von Zellen oder Nukleinsäuren, die von dem Tumor abgestoßen werden, auf Anwesenheit einer inaktivierenden Mutation in *ATRX*;
Zuordnen des ZNS-Tumors zu einer Klasse auf Basis der Anwesenheit der Mutation oder der Identität der Mutation.

- 40 6. Verfahren nach Anspruch 5, wobei der Patient entsprechend der Klasse einer Gruppe für klinische Studien zugeordnet wird.

- 45 7. Verfahren nach Anspruch 5, wobei auf Basis der Klasse eine Behandlung verordnet wird.

8. ATRX-Hemmstoff zur Verwendung bei der Hemmung von ATRX in einem ZNS-Tumor, wobei der Hemmstoff ATRX-Funktion oder -Expression hemmt.

- 50 9. ATRX-Hemmstoff zur Verwendung nach Anspruch 8, wobei der Hemmstoff ein Antikörper, ein Antikörperfragment, ein Einzelketten-Antikörperkonstrukt oder ein inhibitorisches RNA-Molekül ist.

10. Verfahren nach Anspruch 1, 4 oder 5, oder ATRX-Hemmstoff zur Verwendung nach Anspruch 8, wobei der ZNS-Tumor ein glialer Tumor ist.

- 55 11. Verfahren nach Anspruch 1, 4 oder 5, oder ATRX-Hemmstoff zur Verwendung nach Anspruch 8, wobei der ZNS-Tumor ausgewählt ist aus der Gruppe bestehend aus: pädiatrischem Glioblastom, adultem Glioblastom, Oligodendrogliom, Medulloblastom, anaplastischem Oligodendrogliom, Oligoastrozytom, anaplastischem Oligoastrozytom, Astrozytom und anaplastischem Astrozytom.

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12. Verfahren nach Anspruch 1, 2, 4 oder 5, wobei die Mutation ausgewählt ist aus der Gruppe bestehend aus R2079X, Q1874X, Q1788H, E2277K, Q2156H, K455X, W263X, R2153C und R1803H.

13. Verfahren nach Anspruch 1, 4 oder 5, wobei die Mutation ausgewählt ist aus der Gruppe bestehend aus einer Frameshift-Mutation, einer Missense-Mutation, einer Spleißstellen-Mutation und einer Indel-Mutation.

14. Verfahren nach Anspruch 1, 4 oder 5, wobei die Mutation eine große Genomumlagerung ist.

Revendications

1. Procédé pour prédire l'issue d'une tumeur du système nerveux central (SNC) chez un patient, comprenant:

le test de la tumeur du SNC, ou de cellules ou d'acides nucléiques produits par la tumeur, pour la présence d'une mutation inactivante dans *ATRX*, où la mutation est un indicateur de pronostic positif.

2. Procédé selon la revendication 1 où l'étape de test comprend une analyse immunohistochimique.

3. Procédé selon la revendication 1 où l'étape de test comprend une analyse d'acide nucléique.

4. Procédé d'identification d'une tumeur du SNC, comprenant:

le test et l'identification de la présence d'une mutation inactivante dans *ATRX* dans un tissu suspecté d'être une tumeur du SNC, ou dans des cellules ou des acides nucléiques produits par la tumeur, où la présence d'une mutation inactivante indique une tumeur du SNC.

5. Procédé pour classer une tumeur du système nerveux central (SNC) d'un patient, comprenant:

le test de la tumeur du SNC, ou de cellules ou d'acides nucléiques produits par la tumeur, pour la présence d'une mutation inactivante dans *ATRX*
l'attribution de la tumeur du SNC à une classe sur la base de la présence de la mutation ou de l'identité de la mutation.

6. Procédé selon la revendication 5 où le patient est attribué à un groupe d'essai clinique selon la classe.

7. Procédé selon la revendication 5 où un traitement est prescrit sur la base de la classe.

8. Agent inhibiteur d'*ATRX* destiné à être utilisé dans l'inhibition d'*ATRX* dans une tumeur du SNC, où l'agent inhibe la fonction ou l'expression d'*ATRX*.

9. Agent inhibiteur d'*ATRX* destiné à être utilisé selon la revendication 8 où l'agent inhibiteur est un anticorps, un fragment d'anticorps, une construction d'anticorps à une seule chaîne ou une molécule d'ARN inhibitrice.

10. Procédé selon la revendication 1, 4 ou 5, ou agent inhibiteur d'*ATRX* destiné à être utilisé selon la revendication 8, où la tumeur du SNC est une tumeur gliale.

11. Procédé selon la revendication 1, 4 ou 5, ou agent inhibiteur d'*ATRX* destiné à être utilisé selon la revendication 8, où la tumeur du SNC est choisie dans le groupe consistant en: le glioblastome pédiatrique, le glioblastome de l'adulte, l'oligodendrogliome, le médulloblastome, l'oligodendrogliome anaplasique, l'oligoastrocytome, l'oligoastrocytome anaplasique, l'astrocytome et l'astrocytome anaplasique.

12. Procédé selon la revendication 1, 2, 4 ou 5 où la mutation est choisie dans le groupe consistant en R2079X, Q1874X, Q1788H, E2277K, Q2156H, K455X, W263X, R2153C et R1803H.

13. Procédé selon la revendication 1, 4 ou 5 où la mutation est choisie dans le groupe consistant en une mutation par déphasage, une mutation faux-sens, une mutation de site d'épissage et une mutation indel.

14. Procédé selon la revendication 1, 4 ou 5 où la mutation est un grand réarrangement génomique.

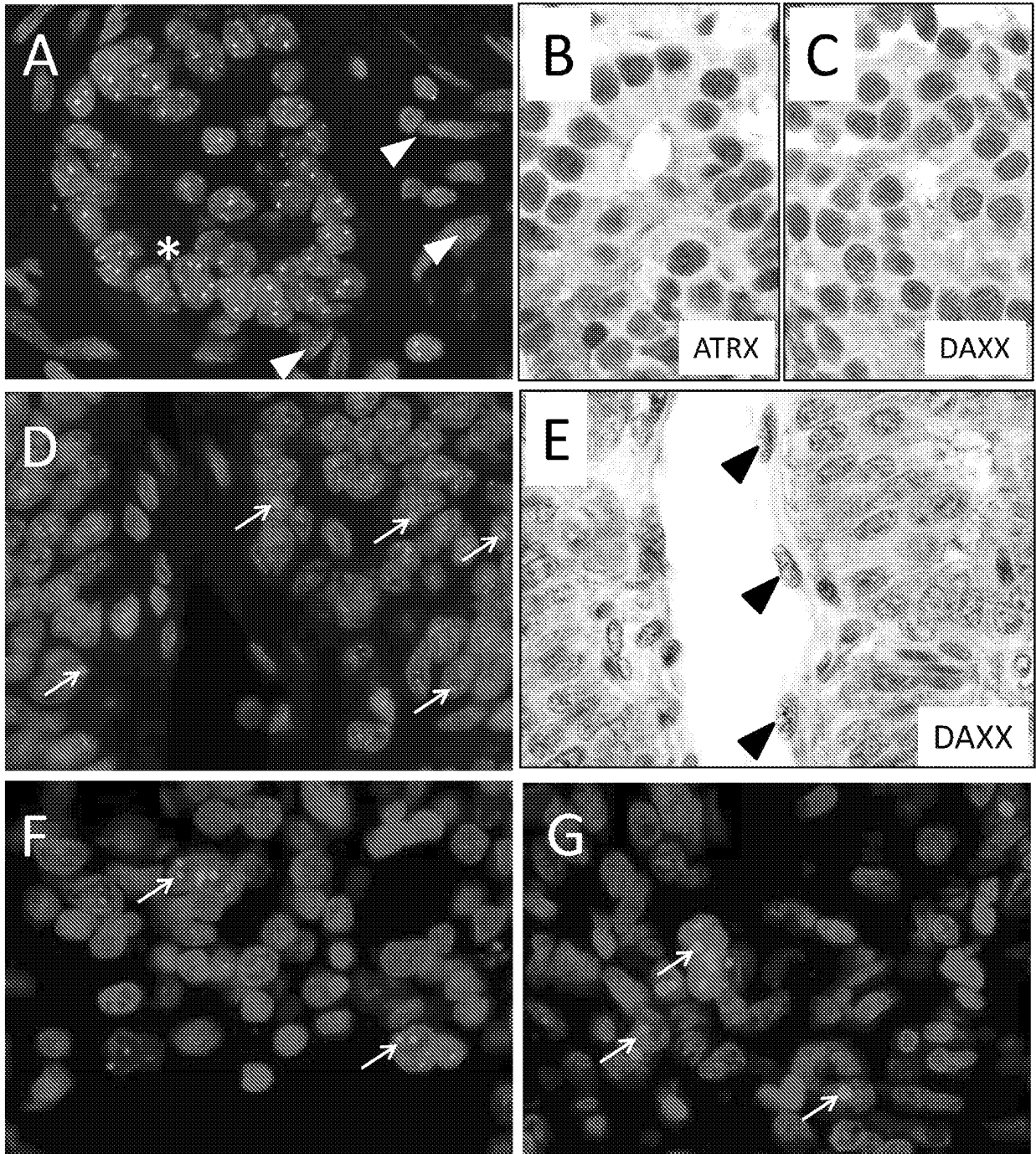


Figure 1A-1G

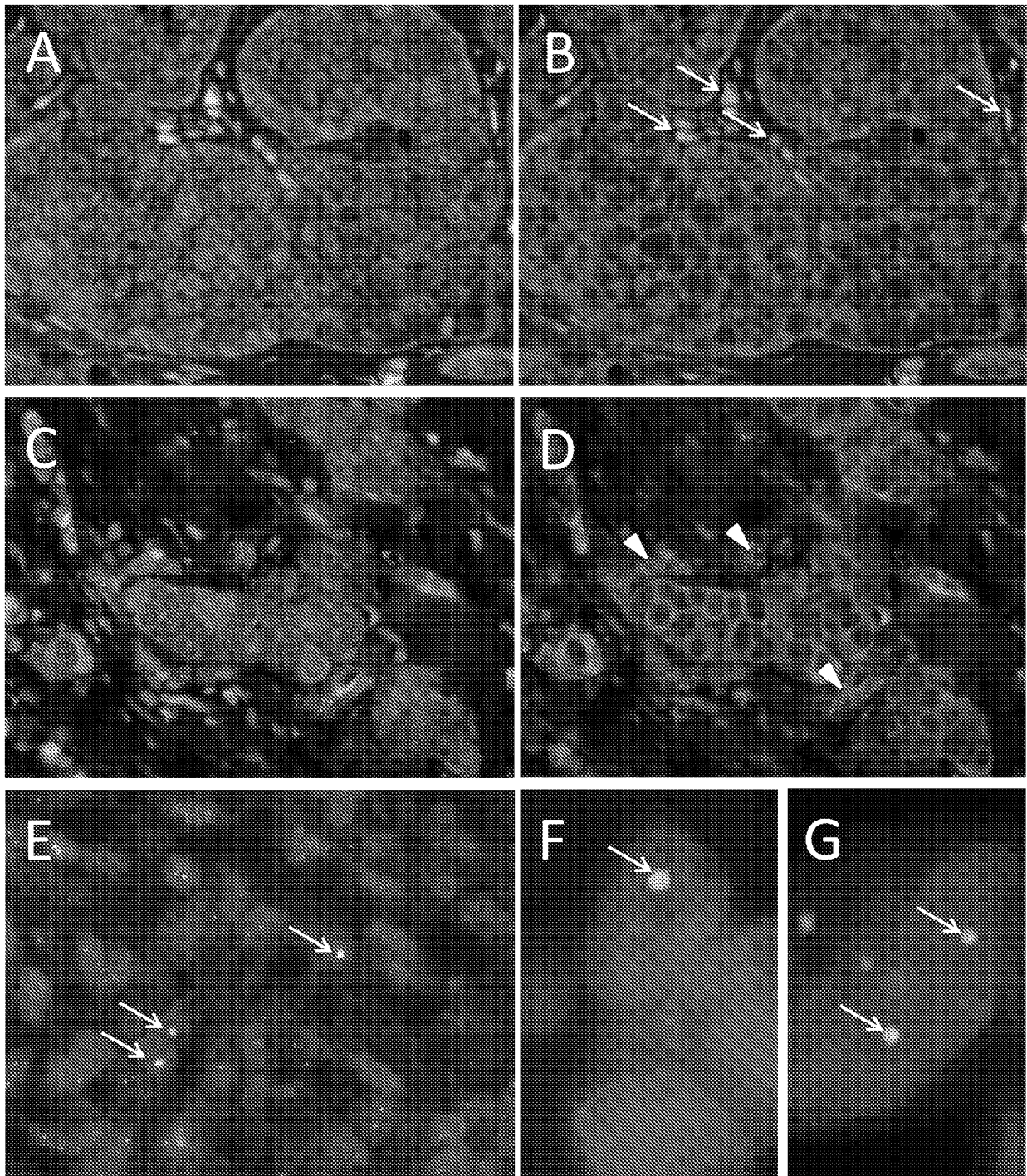


Figure 2A-2G

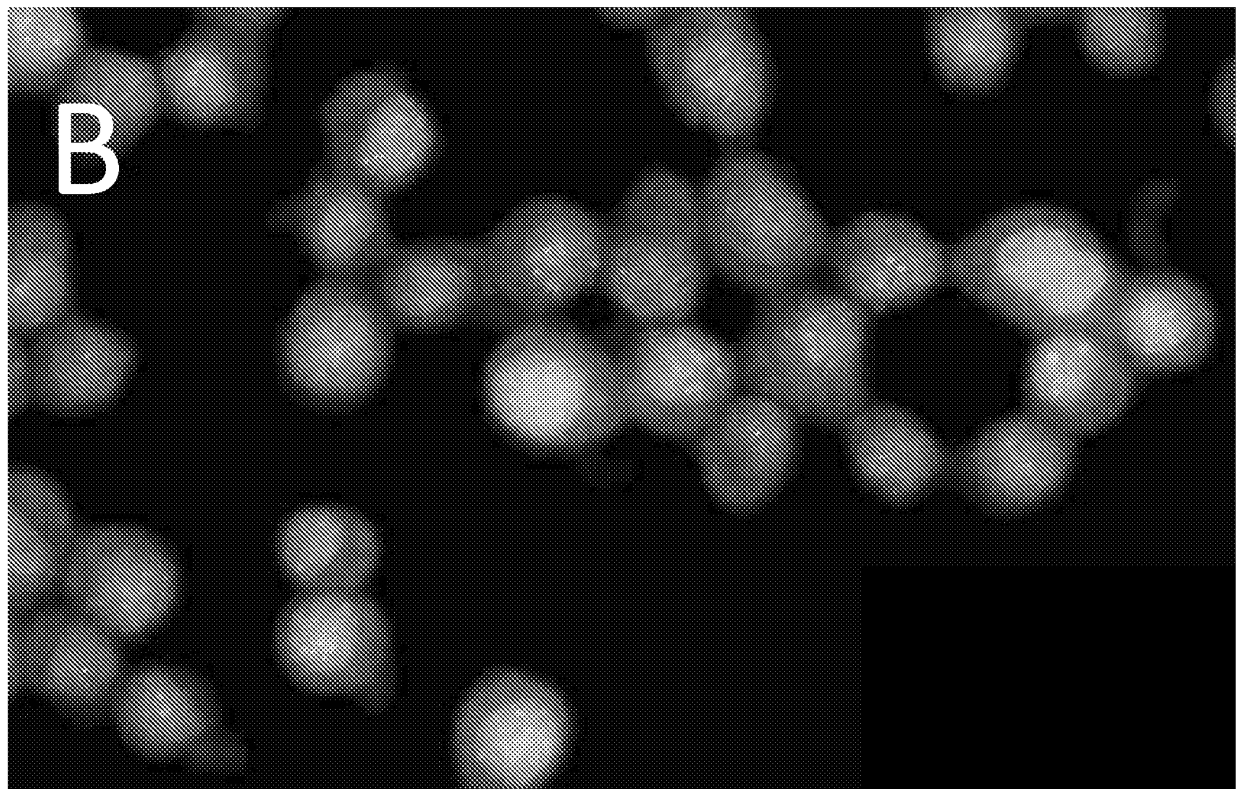
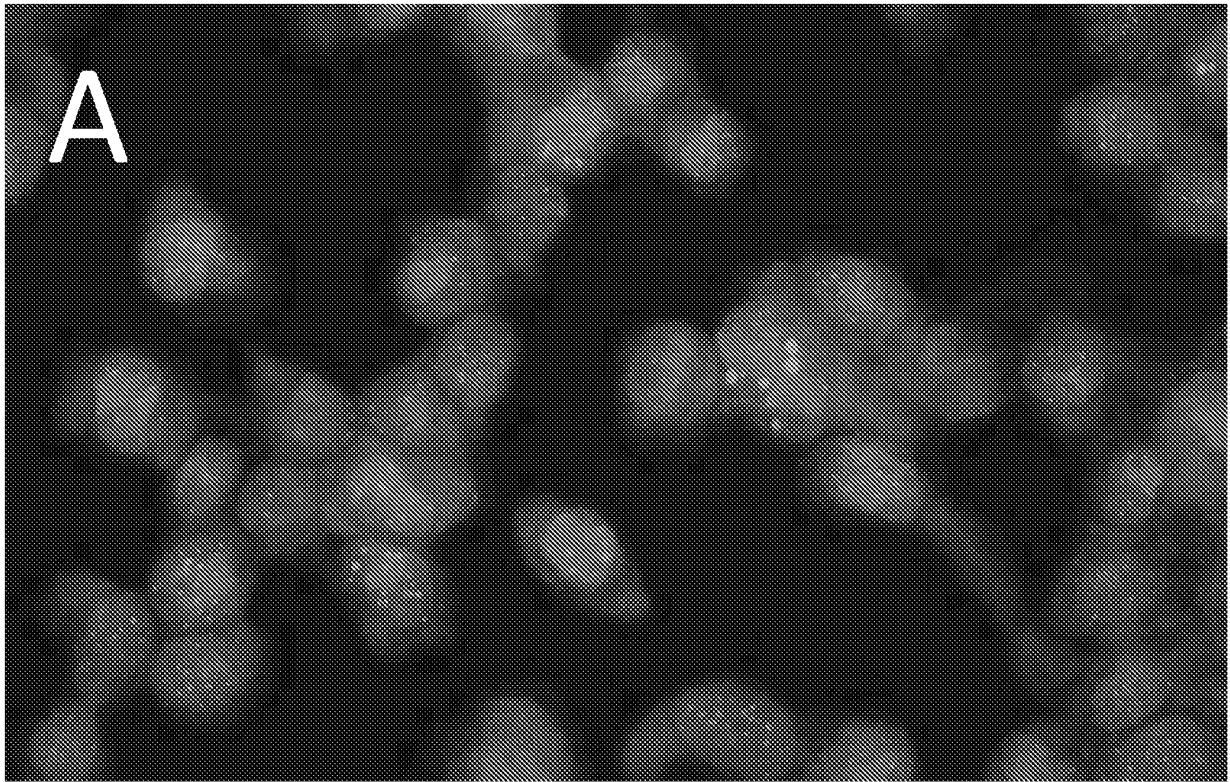


Figure 3 A-3B

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专利名称(译)	脑癌中ATRX的体细胞突变		
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摘要(译)

我们确定了来自不同部位的447种癌症中ATRX和DAXX的序列。我们发现最常见于小儿多形性胶质母细胞瘤 (GBM) (11.1%) , 成人GBM (6.5%) , 少突神经胶质瘤 (7.7%) 和成神经管细胞瘤 (1.5%) 的突变;并且显示端粒替代延长 (ALT) 是一种端粒酶非依赖性端粒维持机制, 在未激活端粒酶的癌症中发现, 与任一基因的体细胞突变完全相关。相反, 神经母细胞瘤和卵巢, 乳腺和胰腺的腺癌对ATRX和DAXX的突变均为阴性。 ATRX或DAXX的改变定义了与人类癌症中替代端粒维持功能密切相关的特定分子途径。

