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SHORT COMMUNICATION

***Braf* Mutations Initiate the Development of Rat Gliomas Induced by Postnatal Exposure to *N*-Ethyl-*N*-Nitrosourea**

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A single dose of *N*-ethyl-*N*-nitrosourea (ENU) during late prenatal or early postnatal development induces a high incidence of malignant schwannomas and gliomas in rats. Although T->A mutations in the transmembrane domain of the *Neu* (*c-ErbB-2*) gene are the driver mutations in ENU-induced malignant schwannomas, the molecular basis of ENU-induced gliomas remains enigmatic. We performed whole-genome sequencing of gliomas that developed in three BDIV and two BDIX rats exposed to a single dose of 80 mg ENU/kg body weight on postnatal day one. T:A->A:T and T:A->C:G mutations, which are typical for ENU-induced mutagenesis, were predominant (41% to 55% of all somatic single nucleotide mutations). T->A mutations were identified in all five rat gliomas at *Braf* codon 545 (V545E), which corresponds to the human *BRAF*V600E. Additional screening revealed that 33 gliomas in BDIV rats and 12 gliomas in BDIX rats all carried a *Braf*V545E mutation, whereas peritumoral brain tissue of either strain had the wild-type sequence. The gliomas were immunoreactive to *BRAF* V600E antibody. These results indicate that *Braf* mutation is a frequent early event in the development of rat gliomas caused by a single dose of ENU. (*Am J Pathol* 2016, 186: 2569–2576; <http://dx.doi.org/10.1016/j.ajpath.2016.05.024>)

N-ethyl-*N*-nitrosourea (ENU) is a potent ethylating mutagen and carcinogen that causes single DNA nucleotide mutations.^{1,2} ENU is one of the most powerful neurocarcinogens in rats³; the frequency and latent period of nervous system tumors depend on the developmental stage at exposure, the dose and method of ENU administration, and the rat strain. In BDIX rats, a single dose of ENU given transplacentally after the 13th day of gestation or early postnatally induces malignant schwannomas (malignant peripheral nerve sheath tumors; in as many as 85%) and gliomas (approximately 30%).^{4–6} ENU-treated BDIV rats develop gliomas at a similar rate as BDIX rats, but they rarely develop malignant schwannomas.⁷

ENU-induced malignant rat schwannomas all contain a T->A transversion at nucleotide 2012, located in the

sequence coding for the transmembrane domain of *Neu* (*c-ErbB-2*).⁸ This mutation was detected as early as 7 days after postnatal ENU administration⁸ and has been considered the driver mutation in the initiation of carcinogenesis.⁹ However, the *Neu* gene is not the target in the induction of gliomas. In this study, we performed whole-genome sequencing and demonstrated that the *Braf* gene is the molecular target for ENU-induced gliomagenesis in rats.

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Q.W. and K.S. contributed equally to this work.

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Materials and Methods

Rat Glioma Tissues

Gliomas induced by a subcutaneous injection of 80 mg ENU/kg body weight on postnatal day 1, in inbred BDIV (3 frozen and 33 formalin-fixed paraffin-embedded samples) and in inbred BDIX (2 frozen and 12 formalin-fixed paraffin-embedded samples) rats,^{10,11} as well as frozen normal brain tissues of one BDIV rat and one BDIX rat were obtained from the Department of Pathology and Neuropathology, University Hospital, Essen, Germany. Both BDIV and BDIX rats have been maintained exclusively by brother × sister mating for >40 generations and are considered as inbred strains with a residual heterozygosity of <0.02%. Genotyping of both strains with >20,000 single nucleotide polymorphism markers by the STAR consortium revealed similar results.¹² The ENU-induced rat gliomas histologically resembled human low-grade or anaplastic oligodendrogliomas, but they lacked diffuse infiltration into normal brain tissues.

Whole-Genome Sequencing in ENU-Induced Rat Gliomas

Frozen samples of ENU-induced gliomas (anaplastic oligodendrogliomas) developed in three BDIV rats and two BDIX rats and frozen samples of normal brain tissues of BDIV and BDIX rats (one each) were used for whole-genome sequencing. For each sample, 22 frozen sections (20 μm thickness) were prepared by cryostat. Presence of >90% glioma cells (<10% normal cells) was confirmed by the first and last sections stained with hematoxylin and eosin. DNA was extracted from the remaining 20 frozen sections using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Quality of DNA was analyzed by Agilent 2200 TapeStation (Genomic Screen Tape and Reagents, P/N: 5067-5366 and 5067-5365) according to the manufacturer's protocol (Agilent, Santa Clara, CA). Genomic DNA was quantified using Qubit 2.0 fluorometer and Qubit ds DNA HS Assay kit (Life Technologies-Invitrogen, Waltham, MA; P/N: Q32851) according to the manufacturer's guidelines. Sequencing libraries were prepared using TruSeq Nano DNA HT Library Prep kit (Illumina, San Diego, CA; P/N: FC-121-4003) according to the manufacturer's instructions and using Agilent NGS Workstation Option B. Final libraries were validated using Qubit and Agilent 2200 TapeStation. From Qubit quantification and size profile analysis, libraries were normalized using Agilent NGS Workstation Option B and clustered on the cBot (Illumina) with a final concentration of 10 to 12 pmol/L (spiked with 1% PhiX control v3; P/N: FC-110-3001) using TruSeq PE Cluster Kit v3 (P/N: PE-401-3001). Paired-end sequencing (2 × 100 bp) was performed using three lanes of a HiSeq2000 (Illumina) each and the 200 cycles TruSeq SBS

Kit v3 (P/N: FC-401-3001) according to the manufacturer's protocols.

Bioinformatic Analysis

Paired-end Illumina sequencing reads were mapped to the rat reference genome rn6 (UCSC Genome Browser assembly ID: rn6; sequencing/assembly provider ID: RGSC Rnor_6.0; assembly date: July 2014; University of California Santa Cruz Genome Browser, <http://hgdownload.soe.ucsc.edu/goldenPath/rn6/bigZips/rn6.fa.gz>, last accessed May 13, 2016) using Burrows-Wheeler Aligner BWA-MEM version 0.7.8,¹³ and then were processed with SAMtools version 0.1.19.¹⁴ Merging and duplicate marking was performed with Picard version 1.125 (Broad Institute, <http://broadinstitute.github.io/picard>, last accessed May 19, 2015). The detailed workflow to call single nucleotide variants (SNVs) has previously been described by Jones et al.¹⁵ In addition, because annotations of read-attracting regions were not available for rn6, we filtered out candidate SNVs in regions where coverage (in 1-kb window) exceeds three times the deviation (median absolute deviation) from the median. The confidence scores for SNV were calculated, as previously reported by Jones et al.¹⁶ During scoring, penalties were given for low sequencing coverage, strand bias among variant-containing reads, low variant allele frequency, and overlapping with simple repeats (RepeatMasker), satellite repeats (RepeatMasker), low complexity (RepeatMasker), or simple tandem repeats (Simple Tandem Repeat Annotation, <http://hgdownload.soe.ucsc.edu/goldenPath/rn6/database/simpleRepeat.txt.gz>, last accessed May 5, 2015). SNVs with high confidence scores were used in the subsequent analysis. Variants were annotated with RefSeq genes (RefSeq Gene Annotation, <http://hgdownload.soe.ucsc.edu/goldenPath/rn6/database/refGene.txt.gz>, last accessed April 30, 2015) using ANNOVAR.¹⁷ Pathway analysis was performed with Database for Annotation, Visualization and Integrated Discovery version 6.7 (<http://david.abcc.ncifcrf.gov>, last accessed Dec 21, 2015).^{18,19}

Short insertions and deletions (indels) were identified using Platypus version 0.7.4 (Wellcome Trust Centre for Human Genetics, Oxford, UK; parameters: genIndels = 1, genSNPs = 0, ploidy = 2, nIndividuals = 2),²⁰ with the tumor and control BAM files as input. Somatic calls (control genotype 0/0) were required to pass either the Platypus filter (flag PASS) or a custom filter allowing low variant frequency. The custom filter used the following scoring scheme. Low-frequency candidates (ie, those with variant allele frequency <10%) were filtered out if they had any of the following three flags: badReads, alleleBias, or strandBias. Candidates were also filtered out if they had any of these three flags plus two or more other flags, or if they had combinations of Platypus non-PASS filter flags, bad quality values, low genotype quality, very low variant counts in the tumor, and variant reads in the control. Indels

were annotated with ANNOVAR, and somatic high-confidence indels occurring in a coding sequence or splice site were considered functional.²⁰

To assess copy number alterations, the coverage across the genome in a 50-kb nonoverlapping window was calculated for each sample, and GC content correction was performed. For a given window, GC content is the fraction of G and C bases in that window according to the rn6 reference genome. The coverage of a window is the total number of reads with a 5'-end in the window. The GC bias curve was determined by the median coverage of all windows with a certain GC content [in steps of 0.01; the median coverage for GC content with too few data points (<20) was not estimated]. Then the coverage of each window was normalized by the point estimate of the corresponding GC content on the GC bias curve. Only autosomes were used for GC bias curve calculation. The GC-corrected coverage data were then smoothed and segmented using DNACopy version 1.36.0 (Memorial Sloan Kettering Cancer Center, New York, NY). The copy number data and the segments were plotted with the Bioconductor package gtrellis (version 1.3.4). Because of the divergence of the BDIV and BDIX strains from the rn6 reference strain, genomic regions where the coverage (in a 1-kb window) was more than three times the median absolute deviation from the mode in the control genome (ie, nontumor) were excluded from the analysis of genomes of the same strain.

Screening of *Braf* Mutations by Sanger Sequencing

Braf V545E mutations were assessed by Sanger DNA sequencing on an ABI 3100 PRISM DNA sequencer (Applied Biosystems, Foster City, CA) with the Big Dye terminator cycle sequencing kit (ABI PRISM; Applied Biosystems) as previously reported^{21,22} in the five rat gliomas whose whole genomes were sequenced. *Braf* V545E mutations were also screened in additional formalin-fixed and paraffin-embedded tissues of ENU-induced gliomas developed in 33 BDIV and 12 BDIX rats.

DNA was extracted from paraffin sections as previously reported.²³ Two sets of primers were used to detect *Braf* V545E mutations. The primer sequences used were *Braf* version 1: 5'-TCATGAAGACCTCACGGTAAAA-3' (sense) and 5'-CCATCCACAAAATAGATCCAGAC-3' (antisense) (PCR product, 110 bp); and *Braf* version 2: 5'-TCATGAAGACCTCACGGTAAAA-3' (sense) and 5'-GAAGAATGCAATCAACTGAGAGAC-3' (antisense) (PCR product, 148 bp).

BRAF V600E Immunohistochemistry

Immunohistochemistry was performed in 10 rat gliomas with *Braf* V545E mutation. On these histologic sections, normal brain (cerebellum) tissues were present and were used as internal negative controls. Paraffin sections were deparaffinized in xylene and 95% ethanol for 5 minutes. After inactivation of endogenous peroxidases with 3% H₂O₂

in methanol for 30 minutes, sections were incubated in preheated phosphate-buffered saline in the oven at 37°C for 10 minutes and then in 0.1% trypsin (Merck, Darmstadt, Germany) in preheated 0.1% CaCl₂ solution at pH 7.8 at 37°C for 15 minutes. Sections were then incubated at 4°C overnight with anti-human BRAF V600E antibody (clone VE1, mouse monoclonal, E19290; Abcys Eurobio, Les Ulis, France), diluted 1:75 or 1:50 in antibody diluent (S3022; Dako, Les Ulis, France). Sections were washed in phosphate-buffered saline and were incubated with the secondary antibody anti-mouse VECTASTAIN Elite ABC kit (Vector Laboratories, Les Ulis, France) for 30 minutes. Visualization was performed using Vector DAB Substrate Kit (SK-4100; Vector Laboratories) for 4 minutes. After washing in phosphate-buffered saline, sections were counterstained with hematoxylin.

Results

Whole-Genome Sequencing

To identify tumor-specific mutations, whole-genome sequencing data of rat gliomas induced by ENU were compared with normal brain DNA of the same inbred strain of rats. The mean number of somatic mutations in the genome was 10,685 (range, 6354 to 13,807) per rat glioma (Figure 1 and Supplemental Table S1). T:A->A:T and T:A->C:G mutations, which are typical for ENU-induced mutagenesis,²⁴⁻²⁶ were 46% to 55% (BDIV) and 41% to 42% (BDIX) of all single nucleotide mutations in rat gliomas detected (minimum coverage, 36.8× for BDIV and 36.2× for BDIX) (Figure 1).

Bioinformatic analysis was first performed to identify functional (miscoding) somatic single nucleotide mutations according to the RefSeq gene annotation, that is, mutations that cause amino acid changes, stop codons, or destroy splice sites. We found 35, 66, and 72 functional somatic mutations in BDIV and 91 and 93 in BDIX rat gliomas. Of these, 92% were missense mutations, 6% nonsense mutations, and 2% splice site mutations. We found seven genes (*Srrm2*, *Olr158*, *Il12rb1*, *Map1b*, *Runx2*, *Rsrc2*, and *Tcf21*) that were mutated in two of the five gliomas analyzed (Supplemental Table S2).

The analysis was then extended to the whole genome, including introns and noncoding regulatory sequences. Only one mutation (chr4:67411708, A->T) was present in all five gliomas analyzed. This mutation appeared to reside in a noncoding region according to the RefSeq (rn6) annotation. However, the University of California Santa Cruz Genome Browser (UCSC Genome Browser assembly ID: rn6; sequencing/assembly provider ID: RGSC Rnor_6.0; assembly date: July 2014)²⁷ revealed that this mutation resides in a region homologous to the coding region of the human *BRAF* and mouse *Braf* genes; this region is annotated as *Braf* proto-oncogene according to the Ensembl gene prediction (Supplemental Figure S1A). These findings

Figure 1 Patterns of somatic single nucleotide mutations in gliomas induced by ENU in rats. The mutation types are on the x-axes, and the y-axes depict the percentage of mutations attributed to a specific mutation type. The total numbers of mutations in each glioma are shown on the top of each panel. Note that T:A->A:T and T:A->C:G mutations, which are typical for ENU-induced mutagenesis, are predominant. ENU, *N*-ethyl-*N*-nitrosourea.

suggested that the mutation occurs in the rat *Braf* gene, which is missing from the RefSeq annotation.

According to the Ensembl gene prediction (Ensembl 81), this mutation results in the nucleotide change GTG->GAG in the transcript (ENSRNOT00000014604.7), which causes an amino acid substitution, from valine to glutamic acid, in the Ensembl protein (ENSRNOP00000014604.7) at position 545. Alignment of the rat sequence around the mutated nucleotide with the homologous human exon, which contains the *BRAF* V600E mutation (T->A), displayed remarkably high conservation of the two regions at both the nucleotide and amino acid levels²⁸ (Supplemental Figure S1B). These observations suggested that this rat mutation is homologous to the human *BRAF* V600E mutation, which is present in a variety of human neoplasms, including tumors of the nervous system.^{29,30}

We also performed whole-genome copy number analyses and small indel detection, but we did not find any recurrent alterations. In one glioma, subclonal copy number alterations were detected in several chromosomes (Supplemental Figure S2). Seven somatic misscoding small indels were detected in three gliomas (Supplemental Table S3).

Screening for *Braf* Mutations in Rat Gliomas by Sanger Sequencing

Braf V545E mutations detected by whole-genome sequencing of five gliomas were confirmed by Sanger sequencing (Figure 2). Further screening of 33 ENU-induced gliomas in BDIV rats and 12 in BDIX rats all carried the identical *Braf* V545E mutation. Normal brain tissues of BDIV and BDIX rats as well as peritumoral brain tissues (n = 16) showed the wild-type *Braf* sequence (Figure 2).

Figure 2 Sanger sequencing shows *Braf* V545E mutation, homologous to human *BRAF* V600E, in a rat glioma induced by ENU. Normal rat brain shows wild-type *Braf* sequence. ENU, *N*-ethyl-*N*-nitrosourea.

BRAF V600E Immunohistochemistry

Immunohistochemistry showed cytoplasmic staining of neoplastic cells in 10 ENU-induced rat gliomas with *Braf* V545E mutation. Glioma cells were immunoreactive to human BRAF V600E antibody, whereas normal cerebellum tissues showed no immunoreactivity (Figure 3). Sections without antibody showed no immunoreactivity.

Pathway Analysis of Functional Genetic Alterations

Pathway analysis using functional mutations detected in the initial analysis (Supplemental Table S2) showed that mutations were enriched in pathways involved in glycosylation site N-linked. Other affected pathways in gliomas in BDIV rats included positive regulation of cell death, positive regulation of apoptosis, and positive regulation of programmed cell death, and those in BDIX rats were G-protein-coupled receptor protein signaling, and neurologic system process.

Discussion

The induction of gliomas and malignant schwannomas in rats by a single perinatal injection of ENU is one of the most striking examples of organ-specific tumorigenesis. Our data demonstrate that the target gene for malignant transformation is *Braf* in glial cells, in contrast to *Neu* in Schwann cells.⁸ Because ENU and its methyl analogue *N*-methyl-*N*-nitrosourea do not require metabolic activation and easily pass the placental and blood-brain barriers,³¹ ENU-induced mutations presumably occur in all organs and cell types. The biological consequences of *Neu* and *Braf* mutations may therefore yield a decisive growth advantage to specific cell types during crucial stages of differentiation.

Similar to *Neu* mutations in rat malignant schwannomas induced by ENU, *Braf* mutations in ENU-induced rat gliomas were T:A->A:T transversions. This is in agreement with several *in vitro* studies that showed that ENU predominantly causes mutations at T:A base pairs.^{24–26} T:A->A:T transversions (50%) and T:A->C:G transitions (35%) were the most frequent in spermatogonial stem cells of *Drosophila* induced by ENU.²⁴ Sequencing of DNA from mouse cells exposed to ENU showed that the predominant types of mutations were T:A->A:T (44%) and T:A->C:G (38%).²⁵ Similarly, T:A base pairs were the main target for mutations at the *hprt* locus of rat skin fibroblasts exposed to ENU, with T:A->A:T mutations being most frequent.²⁶

Predominant mutagenic DNA adducts by ENU include *O*⁶-ethylguanine, *O*²-ethylthymine, and *O*⁴-ethylthymine.^{32,33} *O*⁶-ethylguanine causes C:G->T:A mutations,³⁴ if not repaired by *O*⁶-methylguanine-DNA methyltransferase.³⁵ In human lymphoblasts exposed to ENU, repair rates of *O*²-ethylthymine and *O*⁴-ethylthymine were slow and appear to be independent from *O*⁶-methylguanine-DNA methyltransferase and nucleotide excision repair activities.³³ T:A->A:T mutations were caused by mispairing of *O*²-alkylthymine with thymine during DNA replication,³⁶ whereas T:A->C:G transitions were caused by *O*⁴-alkylthymines.³⁷

In addition to *Braf* mutations, we found 35 to 93 somatic miscoding single base alterations per rat glioma. Except for the common *Braf* V545E mutations, the five rat gliomas analyzed showed different genetic profiles. Thus, even in inbred rats with identical genetic backgrounds exposed to a single dose of ENU, the patterns of genetic alteration differ substantially (Figure 1 and Supplemental Table S1).

We investigated whether there are specific signaling pathways cooperating with *Braf* mutations and found that

Figure 3 A–D: Rat gliomas induced by ENU were diagnosed as low-grade oligodendroglioma (A and B) or anaplastic oligodendroglioma (C and D). Note that oligodendroglioma shows typical honeycomb pattern (B), and anaplastic oligodendroglioma shows brisk mitotic activity (arrows). E–H: Immunohistochemistry shows that glioma cells, but not normal cerebellar granule neurons, are immunoreactive to BRAF V600E antibody. Scale bars: 200 μm (A, C, E, and G); 100 μm (B, D, F, and H). ENU, *N*-ethyl-*N*-nitrosourea.

alterations in genes associated with an N-linked glycosylation site were commonly observed in gliomas of BDIV and BDIX rats. N-linked glycosylation is the most common type of glycosidic bond and important for the folding of certain eukaryotic proteins and for cell-cell and cell-extracellular matrix attachment.^{38,39} In addition, the N-linked glycans of a protein can modulate a protein function, in some cases acting as an on-off switch.³⁸ The possible involvement of this pathway in human glioma development remains to be elucidated. Pathway analyses also showed that there are signaling pathways typical for gliomas in BDIV rats and others typical for BDIX rats.

BRAF is a member of the Raf kinase family of growth signal transduction protein kinases and regulates the mitogen-activated protein kinase/extracellular signal-related kinase pathway, which plays an important role in cell division, differentiation, and development.^{40,41} The *BRAF* gene is mutated in a variety of human neoplasms, with the V600E mutation, which is within the activation segment of the kinase domain, being the most frequent [The Catalogue Of Somatic Mutations In Cancer (COSMIC), <http://cancer.sanger.ac.uk/cosmic>, last accessed June 26, 2016].⁴² *BRAF* V600E mutations have been found in hairy cell leukemia (100%), melanoma (approximately 45%), papillary thyroid cancer (approximately 45%), colorectal cancer (8% to 15%), and non-small cell lung cancer (2% to 5%).²⁹

In the nervous system, *BRAF* V600E mutations have been detected in papillary craniopharyngiomas (96%), pleomorphic xanthoastrocytomas (60% to 100%), gangliogliomas (50%), subependymal giant cell astrocytomas (43%), dysembryoplastic neuroepithelial tumors (30% to 50%), and pilocytic astrocytomas (13% to 18%).^{30,43,44} It is of interest to note that most nervous system tumors in which *BRAF* V600E mutations are frequent or common are non-infiltrative or low infiltrative, and some of these (dysembryoplastic neuroepithelial tumor, ganglioglioma, pilocytic astrocytoma) may contain oligodendroglia-like cells.⁴⁵ These features are similar to those of ENU-induced rat gliomas, which histologically resemble human oligodendrogliomas and anaplastic oligodendrogliomas but lack diffuse infiltration into normal brain tissues (see *Materials and Methods*).

The observation that *Braf* V545E mutations, equivalent to human *BRAF* V600E mutations, are caused by the ethylating carcinogen ENU in rats raises the question of whether some human tumors carrying a *BRAF* V600E mutation may be caused by environmental or endogenous ethylating carcinogens. *N*⁷-ethylguanine, a DNA base lesion caused by ethylating agents, is present in DNA exposed to cigarette smoke.⁴⁶ Smokers have been shown to have higher levels of *N*⁷-ethylguanine in urine and *O*⁴-ethylthymine in lung tissue than nonsmokers.⁴⁷ Kang et al⁴⁸ detected *O*⁴-ethylthymine (0.5 to 140 adducts/10⁷ thymine) in human liver DNA from 15 of 15 autopsy specimens from healthy individuals. Samowitz et al⁴⁹ found a significant association between smoking and a

small subset of colon cancers with *BRAF* V600E mutations. Roden et al⁵⁰ found that the level of tobacco exposure was significantly higher in pulmonary Langerhans cell histiocytosis patients with *BRAF* V600E mutations than in patients without mutations. Another ethylating agent, ethylene oxide, is used as a sterilizing agent⁵¹ and is classified as carcinogenic to humans (associated with lymphoma, leukemia, and breast cancer).⁵² Ethylene oxide was found to induce brain tumors, including gliomas, in rats,^{53,54} although there is no epidemiologic evidence that ethylene oxide is involved in development of human brain tumors.⁵²

In conclusion, the present study demonstrates the presence of a *Braf* mutation, which is equivalent to *BRAF* V600E in humans, in all ENU-induced rat gliomas analyzed, strongly suggesting that this mutation is the driver mutation that initiates gliomagenesis. This opens the door for additional genetic and epigenetic studies on the early stages of ENU-induced malignant transformation of glial cells.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.05.024>.

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