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Expression of *Hqk* Encoding a KH RNA Binding Protein Is Altered in Human Glioma

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The *quaking* gene family encodes single KH domain RNA-binding proteins that play vital roles in cell differentiation, proliferation, and apoptotic processes. The human *quaking* gene, *Hqk*, maps to 6q25–q26, where cytogenetic alterations associated with a variety of human malignancies, including gliomas have been reported. To assess possible relationships of *Hqk* with human diseases such as glial tumors, we first isolated the *Hqk* gene, characterized its structure and expression pattern, and carried out mutational analysis of *Hqk* in primary tumor samples. The *Hqk* gene contains 8 exons spanning a ~200 kb genomic region, and generating at least four alternatively spliced transcripts, *Hqk*-5, *Hqk*-6, *Hqk*-7 and *Hqk*-7B, of which *Hqk*-7 is abundantly expressed in brain. Analysis of primary tumors demonstrated a high incidence of expression alterations of *Hqk* in gliomas (30%; 6/20), but not in other tumors such as schwannomas (0/3), or meningiomas (0/8). Among the tumor samples showing expression alterations, two were devoid of all three major transcripts, one was missing only the *Hqk*-5 message, and only the *Hqk*-7 message was absent in two cases. Our results thus imply the involvement of *Hqk* in human glial tumor progression.

Key words: *quaking* — Gene structure — RNA binding protein — Glioma

The mouse *qkI* gene, encoding a KH domain RNA-binding protein,[1, 2] has been isolated as a candidate gene for *quaking* or *quaking viable* mutation which leads to neurological defects.[3] *Quaking viable* (qk^v)[3] is an autosomal recessive mutation exhibiting hypomyelination phenotype in the central nervous system (CNS).[4, 5] It has been shown that qk^v genome has a large deletion upstream of the *qkI* gene,[1, 2] resulting in reduced expression of the *qkI* in myelin-producing cells of the CNS.[6] *qkI* contains a KH domain, an RNA binding motif originally identified in the heterogeneous nuclear ribonucleoprotein (hnRNP) K.[7, 8] The KH domain is thought to bind directly with cellular RNA.[9] The *qkI* KH domain is embedded in a larger conserved domain of ~200 amino acids, and the whole conserved sequence is called GSG or STAR (signal transduction and activation of RNA) domain.[10] The GSG domain was initially identified by aligning the first three of its family members, *GRP33*,[11] *Sam68*,[12] and *gld-1*.[13] GSG domain family members include *Artemia salina GRP33*, a hnRNP isolated from brine shrimp, *SAM68*, a phosphoprotein involved in *Src* signaling,[14, 15] and *GLD-1*, a protein required for germ cell differentiation in *Caenorhabditis elegans*.[16, 17] Genes homologous to *qkI* were also isolated from *Drosophila*, *Xenopus*, and zebrafish. Mutations in the GSG/STAR domain result in cell proliferation or specific developmental defects in various organisms, suggesting the importance of these genes in development. *Drosophila who/how* gene has been shown to be critical for muscle development[18, 19] and *Xqua* is involved in gastrulation, particularly notochord development.[20] In *C. elegans*, the *GLD-1* protein is required for normal oocyte differentiation as well as sex determination.[13, 16, 17, 21] In mouse, reduction of *qkI* expression results in neurological phenotypes such as body tremor and seizures due to hypomyelination in the CNS,[6, 22] while disruption of *qkI* gene activity leads to embryonic lethality.[23, 24] Sequences homologous to the *qkI* were also found in a human EST database, and were named *Hqk* for human *qk* gene.[25] Although mutation in the *Hqk* gene has not been reported in human, there is evidence that KH domain-containing RNA binding proteins are involved in human diseases. *FMR1*, for example, a gene that encodes a protein containing two KH domains, is responsible for the phenotype of the fragile X syndrome.[26–30] Expression of *KOC*, another KH-RNA binding protein, is elevated in human pancreatic cancer.[31] It is also known that HCC (hepatocellular carcinoma), a homologous protein to KOC, is overexpressed in hepatocellular carcinoma.[32] Furthermore, it was recently reported that one particular protein isoform of the *qkI*, *QKI-7* is a potent inducer of apoptosis.[33] It is thus possible that the *Hqk* gene is involved

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in cell proliferation as well as disease development processes. As an initial step towards understanding the potential roles of *Hqk* gene in disease development, we have isolated both cDNA and genomic clones for *Hqk*, characterized its genomic structure and determined its expression pattern, as well as the chromosomal location of *Hqk*. Possible involvement of *Hqk* in human glial tumors was also investigated.

MATERIALS AND METHODS

Chromosome mapping of *Hqk* The chromosomal location of the *Hqk* gene was determined by PCR amplification of a human/rodent cell radiation hybrid DNA panel (Genebridge 4 hybrid panel; Research Genetics, Huntsville, AL), which consists of 90 hybrid cell clones covering the entire human genome. The DNAs were amplified with primers qk7C (5′-ATGAGGCCAAGAAATTCCATG-3′) and qk5C (5′-CAAAGGCGATTACCAGTTAAC-3′). The products were digested with *Acc*I to distinguish human and hamster sequences. Results of the panel typing was submitted and analyzed at the radiation hybrid mapping page of the Whitehead Institute for Biomedical Research/MIT Center for Genome Research web site (http://carbon.wi.mit.edu: 8000/cgibin/contig/rhmapper.pl). YAC clones containing STS markers adjacent to *Hqk* were isolated. The presence of *Hqk* sequence in these clones were examined by PCR with qk7C and qk5C, or Southern blot analysis with a probe, HQ5K described below.

Cloning and sequencing of cDNA of *Hqk* An oligo-dT primed cDNA was made from 5 μg of total RNA isolated from human fetal heart, brain and adult peripheral blood leukocytes using the SuperScriptII reverse transcriptase (Life Technology, Bethesda, MD). Sequences of human ESTs that showed significant homology with mouse *qkI* were obtained from the EST database of Genebank with the BLASTN search program and were used to design primers for detection of alternative transcripts and filling the gaps of human EST sequence clusters. Primers e2-4 (5′-TGAGCTGCGGAGCCTGCAATAT-3′) and qk7E (5′-TGGTCGTGTTATAACAGCTGC-3′) were used to amplify the coding region for the 7 kb message, and 7-kd (5′-ATGCCAGTCATGCCTGATATT-3′) and qk7B (5′-CATGGAATTTCTTGGCCTCAT-3′) for 3′-UTR of the 7 kb transcript. e2-4 (5′-TGAGCTGCGGAGCCTGCAATAT-3′) and 6CT-1 (5′-TTTCGTTGGGAAAGCCATACC-3′) were employed for amplification of the coding region of the 6 kb message and 6-kd (5′-GGTATGGCTTTCCCAACGAAA-3′) and qk7B (5′-CATGGAATTTCTTGGCCTCAT-3′) for the 6 kb 3′-UTR. Primers e2-4 (5′-TGAGCTGCGGAGCCTGCAATAT-3′) and qk5D (5′-CAAAGGCGATTACCAGTTAAC-3′) were used for amplification of the 5 kb message coding region, while 5-kd (5′-AAAGTTCGAAGGCACGATATG-3′) and *Hqk*5b (5′-CTGTGGGTTAATAGAAACAGC-3′) were utilized for amplification of the 3′-UTR of the 5 kb transcript. Reactions were performed in a Perkin Elmer 9600 thermal cycler using conditions of 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min. PCR products were then cloned into the pGEM-T vector (Promega, Madison, WI) and inserts were sequenced using the PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). For comparative analysis of 5′ and 3′-UTR sequences of *Hqk* and *qkI*, PIP (percent identity plot) analysis was performed with PipMarker (http://bio.cse.psu.edu/).[34]

Determination of *Hqk* genomic structure Human PAC and BAC libraries were screened with mouse *qkI* cDNA probe and positive clones were selected for subsequent analysis. BAC DNAs were isolated with a QIAGEN Plasmid Kit (QIAGEN, Tokyo), completely digested with *Sau*3AI and cloned into a plasmid vector. This minigenomic library was further screened with the 3-XK cDNA probe containing exons 3–5 of mouse *qkI*.[1] Positive clones were identified and inserts were sequenced with T7 and T3 primers to determine exon-intron junctions. Boundaries of exon 5′ and 3′-UTR were determined by direct sequencing of PCR products using one of the PCR primers as a sequencing primer. BAC and PAC clones were also directly sequenced to verify the exon-intron junctions. Distances between exons were determined by measuring sizes of PCR products amplified with primers corresponding to each exon. Briefly, 100 ng of PAC DNA template was amplified with an LA-PCR Kit (TaKaRa Shuzo, Kyoto) in a 50 μl reaction mixture containing 20 pmol of primers, using the following parameters; 94°C, 4 min for denaturation, then 25 cycles of 94°C, 30 s and 65°C, 12 min. Identities of the PCR products were verified by Southern blot hybridization or DNA sequencing. Sizes of introns longer than 20 kb were determined by restriction mapping of genomic fragments. The exon/intron boundaries and the size of introns except for intron 1 were also confirmed by comparing the data with genomic sequence data recently uploaded at the Sanger Center (http://www.sanger.ac.uk/HGP/Chr6/Chr6_blast_server.shtml).

RT-PCR and northern blot analyses To look for expression of *Hqk* in human embryonic tissue samples, total RNAs were isolated from human fetal heart and brain (18-week) with Trizol reagent according to the manufacturer's instructions (Life Technology, Bethesda, MD). For amplification of the 7 kb message cDNA, primers EH5-3 (5′-GAAGATGCAGCTGATGGAG-3′) and qk7E (5′-GCAGCTGTTATAACACGACCA-3′) were used. The PCR product size was 456 bp. The 492 bp RT-PCR product derived from the 6 kb message were generated with primers EH5-3 (5′-GAAGATGCAGCTGATGGAG-3′) and

6CT-1 (5′-TTTCGTTGGGAAAGCCATACC-3′). For the 5 kb message cDNA, EH5-3 (5′-GAAGATGCAGCT-GATGGAG-3′) and qk5D (5′-CAAAGGCGATTACCA-GTTAAC-3′) were used to obtain a PCR product of 604 bp. PCR products were cloned into the pGEM-T vector (Promega) and inserts were sequenced.

Human multiple tissue northern (MTN) blots (Clontech, Palo Alto, CA) were used for northern analysis with HQ2-6, a probe containing exon 2 to exon 6 that includes the conserved KH domain. HQ2-6 was made by amplification of human cDNA with primers e-3 (5′-CGGAAAGACAT-GTACAATGAC-3′) and e6-u (5′-GCATGACAGCG-GTCTGTATTT-3′). The same blots were rehybridized with other probes corresponding to different alternative transcripts. Probes specific to the alternative transcripts were made by amplification of the *Hqk* cDNA. For the 5 kb transcript, a probe named HQ5K (Fig. 2B) was amplified using primer pair qk5a (5′-GTTCGTCTTACCAT-CTAA-3′) and qk5b (5′-AAGCATGGCTTTTACATC-CT-3′). For the 7 kb transcript, a specific probe named HQ7K (Fig. 2B) and primer pair 7-kd (5′-ATGCCAG-TCATGCCTGATATT-3′) and qk7H (5′-TGTGCAATA-GAATACAGCCTC-3′) were used. A Fuji BAS2000 imaging analyzer was used to analyze the hybridization signals.

Expression analysis and mutation screening of *Hqk* in human tumor samples We performed PCR amplifications of genomic DNA from a panel of primary tumors including twenty samples of glioma, eight meningioma samples and three schwannoma samples. For all the human samples used in this study, informed consents were obtained. Tumor tissues were taken from surgical operations conducted at the Kumamoto University Hospital and processed by the standard methods as described by Miyakawa *et al*.[35] Each tumor specimen was first examined pathologically, and the specimens containing a high percentage of tumor cells, generally more than 95%, were used for the subsequent molecular studies. DNA extraction from the tumor tissues was performed as described by Liang *et al*.[36] Peripheral blood samples were obtained from patients with glial tumors, and DNAs were extracted with the DNA Extractor WB Kit (Nippon Gene, Toyama) according to the protocol provided by the supplier. To search for mutation in *Hqk* of these patients, each exon of the *Hqk* gene was amplified from genomic DNAs of both tumor and blood samples. Primers used for amplification of each exon are summarized in Table I. Sequences of the PCR products were determined by direct sequencing. For Southern analysis, probe HQAX-1, a 349 bp *Apa*I-*Xho*I genomic DNA fragment containing exon 1 was used. Probes HQ2-6 and HQ5K were also used for the Southern blot analysis.

To look for alteration in *Hqk* expression in human tumor samples, total RNAs were isolated from tumors with Trizol reagent according to the manufacturer's instructions (Life Technology). Five micrograms of total RNA was used for first-strand cDNA synthesis using a cDNA preamplification kit (Life Technology). RT-PCR was performed with the same cycling parameters as described for genomic DNA PCR. Primers used were e2-4 and qk7E for the 7 kb message, e2-4 and 6CT-1 for the 6 kb message, and e2-4 and qk5D for the 5 kb message. Primer sequences were as described above. PCR products were cloned into the pGEM-T vector (Promega) and inserts were sequenced. Some of the tumor samples were analyzed by northern blot hybridization using probes described above.

The nucleotide sequence data reported in this paper have been submitted to DDBJ and have been assigned the accession numbers AB067798 (human *quaking* gene, 5 kb mRNA), AB067799 (6 kb mRNA), AB067800 (7 kb mRNA), AB67801 (7 kb-B mRNA); AB067802 (exon 1), AB067803 (exon 2), AB067803 (exon 3), AB067804 (exon 4), AB067805 (exon 5), AB067806 (exon 6), AB067807 (exon 7), AB067808 (exon 8).

RESULTS

Chromosome mapping of *Hqk* The chromosomal location of the *Hqk* gene was determined by use of a Gene

Table I. Details of the Primer Sequences Used to Amplify the Individual Exons of the *Hqk* Coding Region

Exon No.	Forward primer	Reverse primer	Product size (bp)
2	ATGATAGAATAGGCCAGGAG	CACACTTCAGTTGATGACTGC	412
3	TTCTAGCTGTATACTGTTCCC	AAAGTGCTGGGATTACAGGCG	505
4	TTCAATCAAACTTTGAGGACCC	TTAGAAGTATCAGTCAGGTG	459
5	AGGCTCCATTATGTTAGCACC	AACAAGATAAGCTGCAAGCCC	576
6	GCATTTTTGTGTGATCAGCGC	ACTGAACCTCAGTTACCAAAGTG	673
7a	AAATACAGACCGCTGTCATGC	TGGTCGTGTTATAACAGCTGC	1203
7b	AAACAGATGCAGACATGTGTG	TGCAAGTAAGTCACTGACTGC	242
7c	AAACTACTGTGCCTTAACGTG	TTCCATCAGGTGTGGTGTTGC	294
8	ATGAGGCCAAGAAATTCCATG	CAAAGGCGATTACCAGTTAAC	748

bridge 4 radiation hybrid panel. The *Hqk* locus should be located on chromosome 6 in the region between 6q25 and 26 flanked by markers CHLC.GATA81B01 and WI-4442, as shown in Fig. 1. YAC clones positive for these STS markers were isolated, and examined for the presence of the *Hqk* sequence by PCR or Southern hybridization. These analyses confirmed that *Hqk* was indeed contained in the YAC clones 837-D-9 and 920-A-3 (Fig. 1).

Cloning of human homolog of the *qkI* gene, *Hqk* Zorn *et al.*[25] previously reported that sequences homologous to the mouse *qkI* could be retrieved from a human EST database. In order to obtain more complete information on *Hqk* cDNA sequences, we again searched the current human dbEST database. A total of 41 human EST sequences showed significant similarities to the mouse sequences, and were classified into 14 clusters. For example, an EST sequence (Acc# EB893920) showed strong similarities to the exon 2 sequences of *qkI*, which encodes the amino-terminus of *qkI*, while other ESTs were homologous to the carboxyl-terminus of the *qkI* gene product. To fill gaps between the clusters and to obtain entire sequences for all alternative transcripts, we amplified cDNA templates derived from human RNA using primers specific for 5 kb, 6 kb, and 7 kb alternative messages, and sequenced the products to ensure that they were indeed derived from the *Hqk* gene. We obtained the entire coding sequence and a portion of the 3′-UTR sequence for each alternative transcript. The predicted open reading frame is 1023 nucleotides in length for the 5 kb message, 957 nucleotides for the 6 kb message, 975 nucleotides for the 7 kb transcript and 1059 nucleotides for the 7kb-B message. The sequences showed strong similarities to the mouse *qkI* gene.[2] The two genes are highly homologous to each other; the amino acid sequence of each isoform of *Hqk* is identical to that of mouse *qkI*, and 96% identity was found even at the nucleotide level. Given the high homology between the human and mouse *qk* gene, it is likely that the ATG triplet found at nucleotide 553 in the human sequence (Acc#: AB067798) represents a start codon of the human *Hqk* gene. Genomic fragments upstream to the start codon were isolated from the *Hqk*-positive BAC clone and a sequence of about 4 kb was obtained. The region approximately 800 bp region upstream to the start codon was highly similar to that of *qkI*, which corresponds to the putative intron 1 and a part of exon 2 of the *qkI* gene. Similarity in this 800 bp region is nearly 80%, while the homology sharply drops to ~50% in the further upstream region. PIP analysis showed that this region is CpG or GpC-rich (see Fig. 2B). We have not precisely mapped the transcription initiation site yet. However, it is likely that the 5′-UTR is no larger than 500 bp, since the predicted message size is comparable with the length of the cognate transcript estimated by northern blot hybridization. PIP analysis also showed that the 3′-UTR regions of *Hqk* were very similar to the corresponding mouse 3′-UTR sequences. The 3′-UTR nucleotide identities between human and mouse are 85%, 73% and 73% in the 5 kb, 6 kb and 7 kb transcripts, suggesting that the 3′-UTRs may share similar gene-regulating functions among species.

Exon-intron organization of *Hqk* To determine the

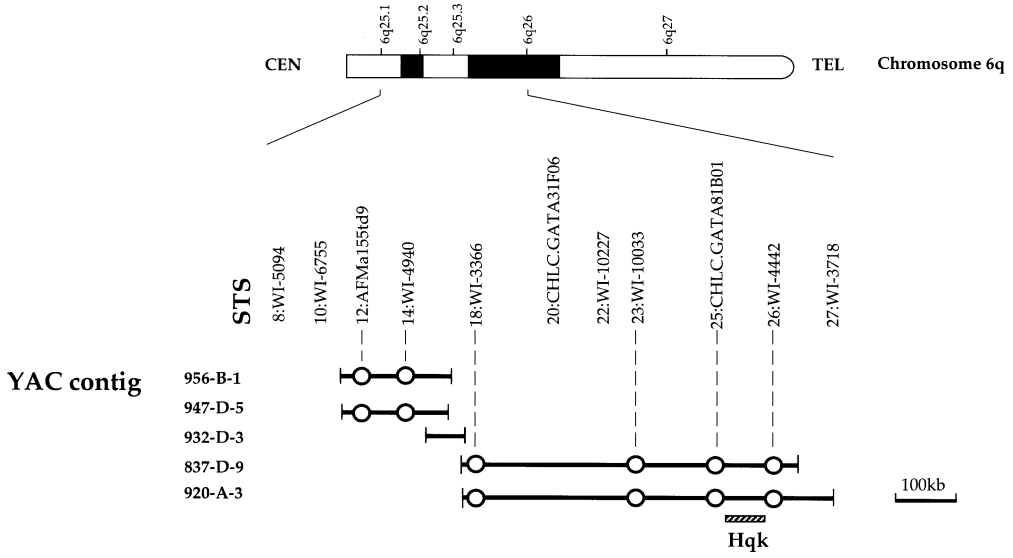

Fig. 1. Chromosomal location of *Hqk* gene. Location of the *Hqk* was determined by typing a Gene bridge 4 radiation hybrid panel. Top: long arm of human chromosome 6. Middle: STS markers in the vicinity of *Hqk*. The *Hqk* gene was mapped between the markers CHLC.GATA81B01 and WI-4442. Bottom: a YAC contig covering the *Hqk* genomic region. Each bar represents a YAC clone and open circles indicate that the YAC clone contains the corresponding STS markers. It was confirmed by Southern analysis that the clones 837-D-9 and 920-A-3 contained the *Hqk* gene.

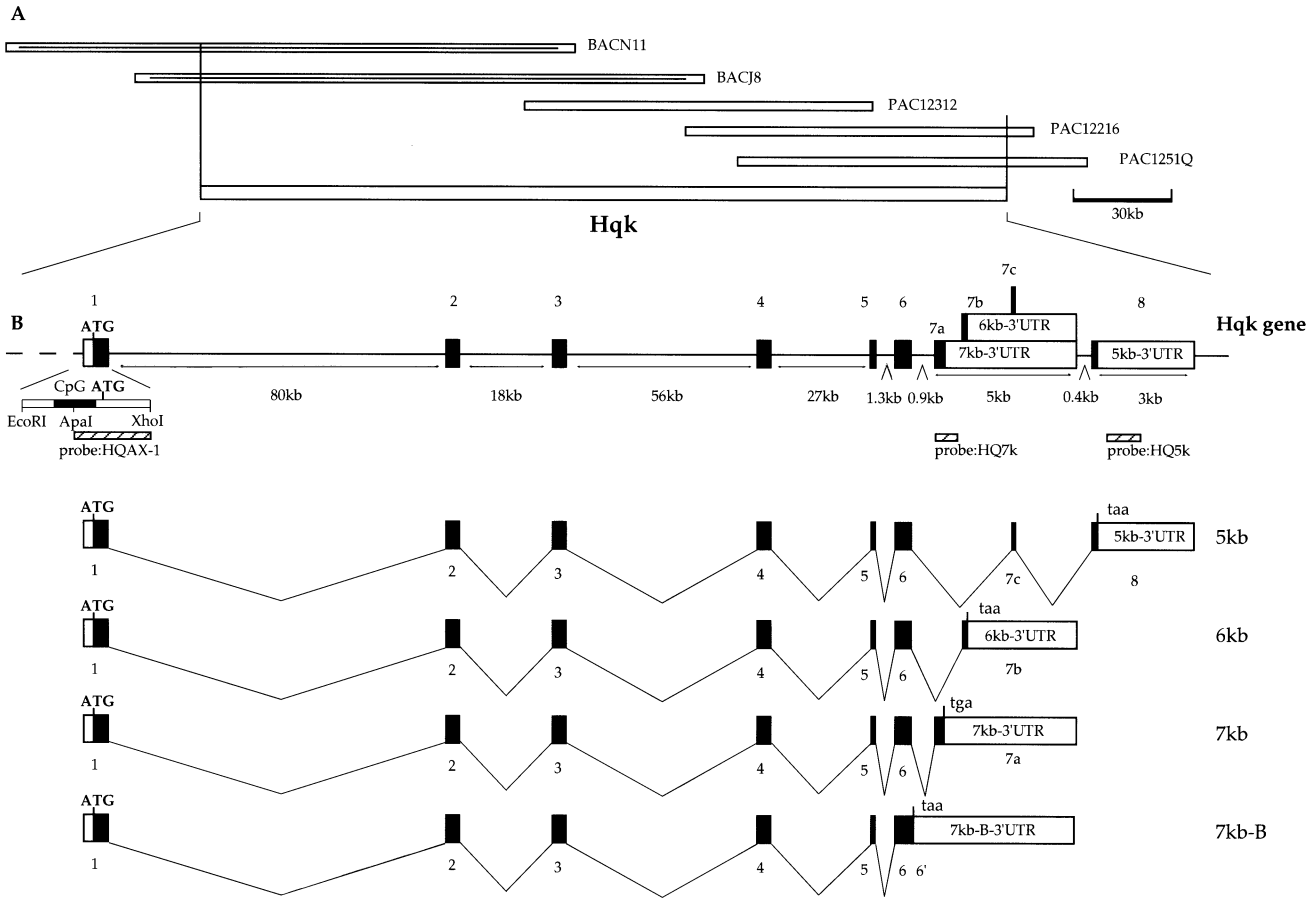

Fig. 2. Genomic organization of the *Hqk*. A) A BAC/PAC contig which covers the entire *Hqk* locus. B) Genomic organization of the *Hqk* gene. The *Hqk* gene consists of eight exons, of which exon 7 is differentially utilized for each alternative transcript. Boxes indicate exons, and closed boxes show coding exons. The 5′-UTR may be larger than the one shown in this figure. CpG islands were found in the region upstream to the translation initiation site. Sizes of introns are shown in kb. Locations of the probes used for Southern blot analysis are also shown. Splicing patterns for each alternative transcript, i.e. 5 kb, 6 kb, 7 kb and 7 kb-B, are shown at the bottom panel.

genomic organization of the *Hqk* locus, human PAC or BAC libraries were screened with a mouse *qkI* cDNA probe containing the conserved GSG/STAR domain. We isolated three PAC and two BAC clones, which covered approximately 300 kb of a genomic region including the *Hqk* locus (Fig. 2A). We constructed a library of short genomic DNA fragments derived from the PAC and BAC clones, and clones containing *Hqk* exons were selected and sequenced to determine exon-intron junctions. These clones were found to contain exons 2–4. By compiling such genomic analysis data, the exon-intron organization of the *Hqk* was determined to be as shown in Fig. 2B. *Hqk* contains eight coding exons and two distinct UTRs spanning a genomic region of approximately 200 kb with large introns of 80 kb between the first and the second exon and of ~56 kb between the third and fourth exon (Fig. 2B). The size of the *Hqk* locus is thus much larger than that of the mouse *qkI* locus, which is about 70 kb, and the entire locus was covered by three BAC/PAC clones (Fig. 2A). This size difference arises from differences in intron length between the two species. The exons range in size from 89 bp (exon 5) to 5687 bp (exon 7), and all of the exons possesses proper splicing donor/acceptor sequences (Table II). However, utilization of the seventh exon is unusual; the exon is used differentially for each alternative transcript (Fig. 2B). For the 7 kb transcript, the entire 5687 bp sequence is used as the exon, of which the first 41 bases represent a coding exon followed by a termination codon, tga, and a 3′-UTR of 5438 bp in size. For the 6 kb message, the first 1329 bp of exon 7 is spliced out, and a smaller exon of 4200 bp containing 23 bp coding exons and a 4177 bp 3′-UTR is present. For the 5 kb message,

Table II. Exon-intron Boundary Sequences of the *Hqk* Gene

Intron sequence	Exon number and sequence	Intron sequence	Exon size (bp)
	---------- 1---- CTGCTGGACGAAG	**gt**gagcgtctccaggg	ND
ttttactttttaac**ag**	AAATTA -- 2---- AGAATACCCAGAT	**gt**aagtatcatgttca	144
ttttttttttctc**ag**	TTTAAT --- 3---- GGATAAAAAAAAAG	**gt**aagtccttgaaaat	117
tctgtaaatttttt**ag**	GAGGAG-- 4---- GGTACCTCCTGCA	**gt**aagtaataatttcc	144
tgttttccatgtat**ag**	GCAGAA -- 5---- AACTTTAAATCAC	**gt**aagaatgagctctg	88
tctttgcttactgt**ag**	CAGCCC -- 6---- AGTGGTGTATTAG	**gt**aagttcttctcccc	300
tgccttttttttat**ag**	AGTGGA -- 7a---TATTTC**TGA**CT--	———	*5687(*41*+5646)
ttccttttatcttt**ag**	GTATGG -- 7b---AAAGGC**TAA**GA--	———	*4200(*23*+4177)
attgcaatttaact**ag**	GTGCGG -- 7c---ACCGCAGACCGAG	**gt**tagtttagttctgc	75
gtttctaaccaccc**ag**	CCGCCA -- 8---- CGGAAC**TAA**CC--	———	*3183(*14*+3169)

Consensus sequence for splice acceptor and donor sites are shown in bold face. The stop codons are underlined. ND, not determined.
∗Exon 7a, 7b and 8 are the last exons of the 7 kb, 6 kb, and 5 kb alternative transcripts, respectively. Sizes of the coding sequences in these exons are italicized and shown in parentheses.

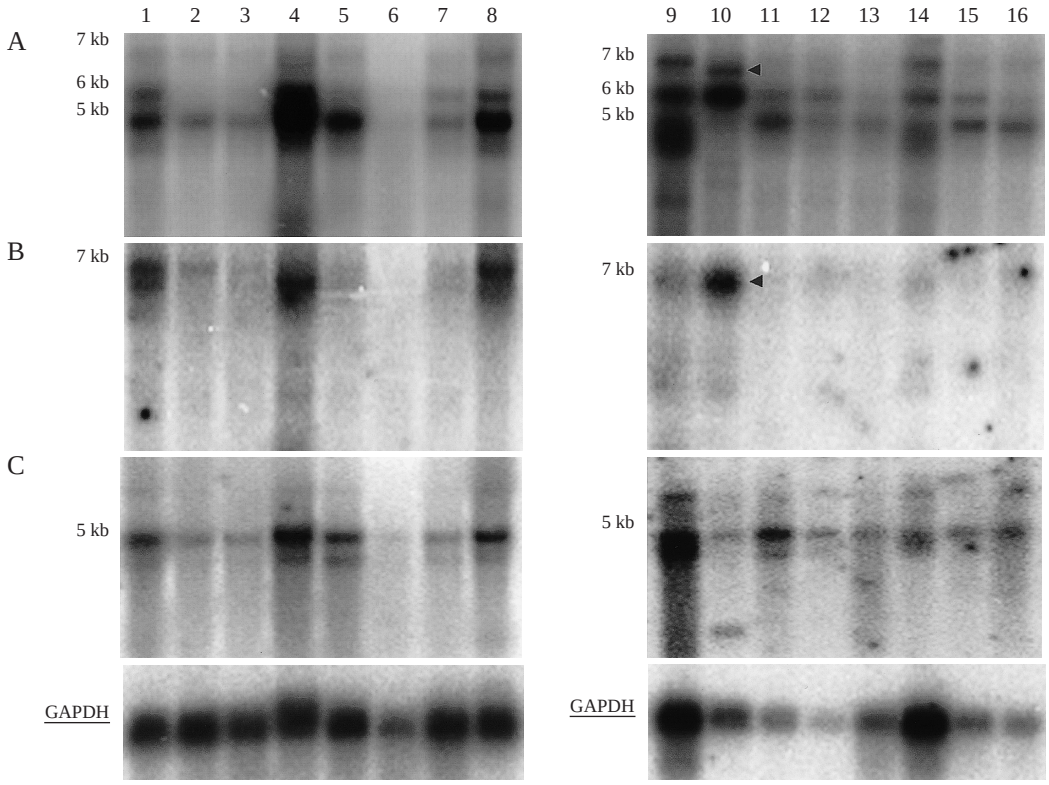

Fig. 3. Northern blot analysis of *Hqk* expression in various human tissues. Human multiple tissues northern blots were hybridized separately with a *Hqk* cDNA probe containing the conserved KH domain (A), a probe specific for the 7 kb message as well as 7 kb-B message (B), or a 5 kb message-specific probe (C). Poly A^+ RNAs (2 μg/lane) from spleen (lane 1), thymus (lane 2), prostate (lane 3), testis (lane 4), ovary (lane 5), small intestine (lane 6), colon (mucosal lining) (lane 7), peripheral blood leukocytes (lane 8), heart (lane 9), brain (lane 10), placenta (lane 11), lung (lane 12), liver (lane 13), skeletal muscle (lane 14), kidney (lane 15), and pancreas (16) were loaded. Integrity and amount of RNA loaded onto the northern blots were examined by probing with a glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) cDNA. The position of the 7 kb message is indicated by triangle in lane 10 of panels A and B.

only a 75 bp sequence of the exon 7 (exon 7c) is utilized as a part of the coding exons. The first 14 bp sequence of exon 8 belongs to the coding region of this message. In the case of the 7 kb-B, intron 6 is not spliced out, resulting in a C-terminal amino acid sequence distinct from that of the other three protein products (data not shown; see Acc# AB067801). Apart from the intron length difference, the genomic structure of *Hqk* is similar to that of the mouse *qkI*. However, there are minor differences in exon-intron organization between the two. For example, the last 69 bp of the mouse exon 5 corresponds to the upstream part of a novel human exon designated as 5 and the first 20 bp of the mouse exon 6 is homologous to that of the human exon 5 (Fig. 2B). From the *Hqk* locus, at least four alternative messages encoding four different protein products are generated. All of the four alternative transcripts can be found in mice as well.[2)]

Expression of *Hqk* messages in human tissues The expression of *Hqk* was examined by northern blot analysis and RT-PCR. We detected the transcripts of *Hqk* in fetal heart and brain tissues by RT-PCR (data not shown), suggesting the possibility that *Hqk* may play an important role in development, as the *qkI* does in mouse. With a probe containing the highly conserved KH domain sequence, bands with several different sizes were detected on northern blots. Signals were found in most of the tissues examined, except for duodenum. On the other hand, three major transcripts of approximately 5, 6, and 7 kb in size were detected in a variety of human tissues (Fig. 3), probably corresponding to the 5, 6 and 7 kb messages in mouse. In some of the tissues examined, there were also bands with sizes different from the 5, 6, and 7 kb transcripts, which might represent uncharacterized alternative transcripts. Based on the knowledge of *Hqk* genomic organization, we designed probes specific to each transcript and used them separately for northern analysis (Fig. 3, B and C). There were some variations in relative expression level of the alternative transcripts among different tissues. For example, the 5 kb message was abundant in heart, but scarcely expressed in brain. The situation was opposite for the 7 kb message. When hybridized with the probe specific to the 7 kb and the 7 kb-B messages, it was found that a band of about 7 kb was strongly expressed in brain, while only a weak band was detected in heart. Compared to the 5 kb

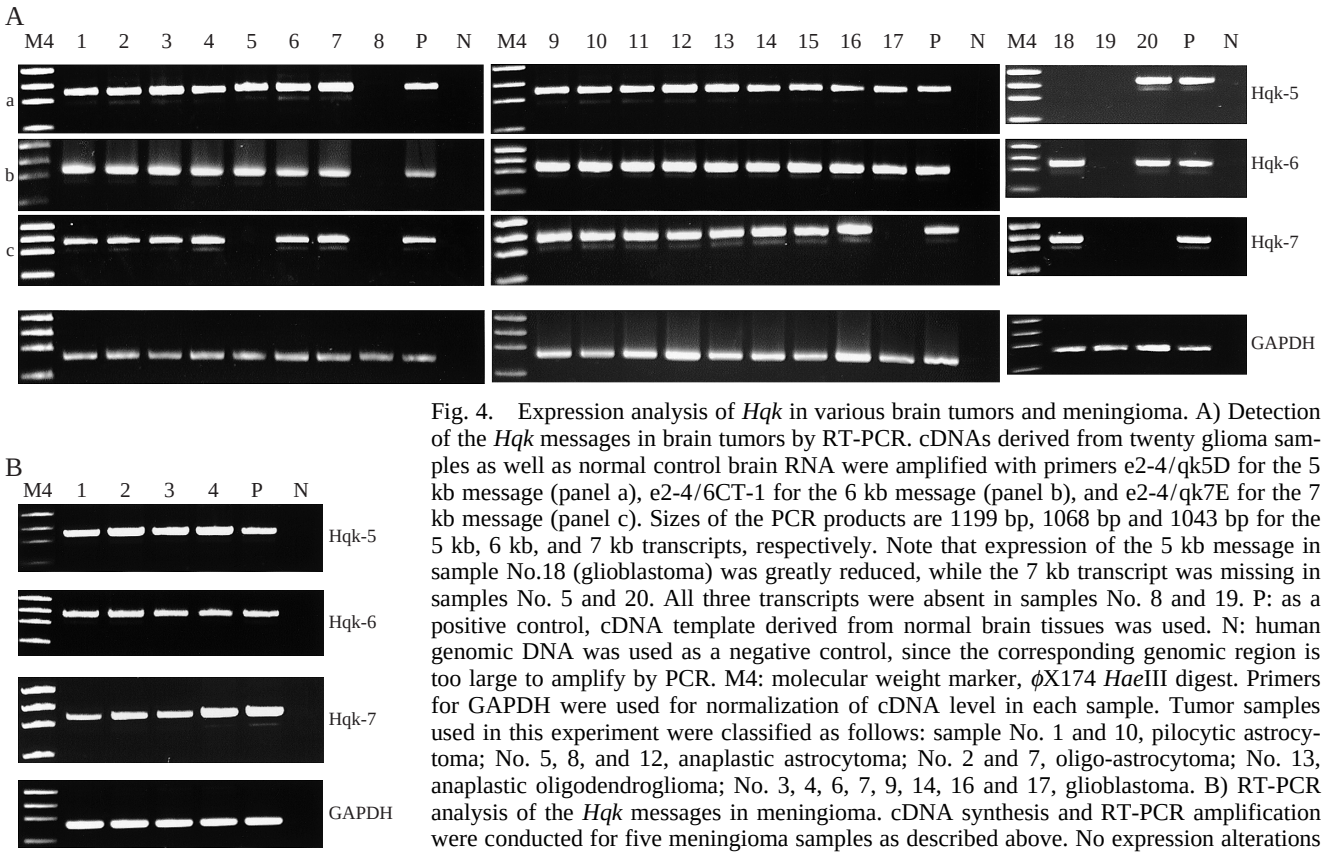

Fig. 4. Expression analysis of *Hqk* in various brain tumors and meningioma. A) Detection of the *Hqk* messages in brain tumors by RT-PCR. cDNAs derived from twenty glioma samples as well as normal control brain RNA were amplified with primers e2-4/qk5D for the 5 kb message (panel a), e2-4/6CT-1 for the 6 kb message (panel b), and e2-4/qk7E for the 7 kb message (panel c). Sizes of the PCR products are 1199 bp, 1068 bp and 1043 bp for the 5 kb, 6 kb, and 7 kb transcripts, respectively. Note that expression of the 5 kb message in sample No.18 (glioblastoma) was greatly reduced, while the 7 kb transcript was missing in samples No. 5 and 20. All three transcripts were absent in samples No. 8 and 19. P: as a positive control, cDNA template derived from normal brain tissues was used. N: human genomic DNA was used as a negative control, since the corresponding genomic region is too large to amplify by PCR. M4: molecular weight marker, ϕX174 *Hae*III digest. Primers for GAPDH were used for normalization of cDNA level in each sample. Tumor samples used in this experiment were classified as follows: sample No. 1 and 10, pilocytic astrocytoma; No. 5, 8, and 12, anaplastic astrocytoma; No. 2 and 7, oligo-astrocytoma; No. 13, anaplastic oligodendroglioma; No. 3, 4, 6, 7, 9, 14, 16 and 17, glioblastoma. B) RT-PCR analysis of the *Hqk* messages in meningioma. cDNA synthesis and RT-PCR amplification were conducted for five meningioma samples as described above. No expression alterations were found in these non-glioma samples.

message, the 7 kb message showed a more restricted expression pattern. To look for transcripts which were undetectable by northern analysis, RT-PCR was carried out. Based on the genomic structure of the mouse *qkI*, primers were designed to distinguish six alternative transcripts in human, if they exist. In addition to the major transcripts of the 5, 6, and 7 kb messages, a novel transcript designated 7 kb-B were detected, while other minor transcripts found in mice were not detected in human fetal heart, brain, and adult leukocytes (data not shown). These data suggest that three major alternative transcripts are produced from the *Hqk* locus, and that their expression levels vary among the human tissues tested.

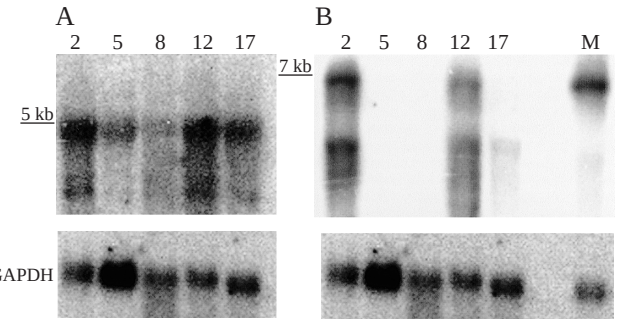

Fig. 5. Northern analysis of *Hqk* expression in brain tumor samples. For the seven glioma samples, in which *Hqk* expression alterations were detected by RT-PCR, northern blot analysis was further conducted. Seven micrograms of total RNA extracted from the tumor samples was loaded, and probed with the 5 kb specific probe (panel A) or the 7 kb probe (panel B). In lane M of panel B, mouse brain RNA was loaded as a positive control. The 7 kb probe detected the 7 kb message of mouse *qkI* and the 7 kb *Hqk* band in samples 2 and 12, while the 7 kb message was absent in samples #5, 8 and 17. The lower band in lane 2, 12 or 17 probably represents cross hybridization with 28S ribosomal RNA. Hybridization results with the GAPDH probe (lower panel) revealed that similar amounts of RNA were loaded in all lanes.

Alterations of *Hqk* expression in human tumors Cytogenetic studies suggest a high incidence of genomic deletion in the long arm of human chromosome 6 in various tumors including glioblastomas and astrocytomas.[35–37] To investigate whether *Hqk* is involved in tumor formation, we first looked for genomic deletion of *Hqk* in tumor samples including twenty gliomas, eight meningiomas and three schwannomas. PCR primer pairs that flank each exon were used to detect homozygous genomic deletions, but no such deletions were found in the tumor samples tested. Point mutations that would affect amino acid sequence or splice-site consensus sequences were not found in the PCR products. Genomic Southern blot analysis also failed to detect deletions in the *Hqk* locus. Then, we examined the *Hqk* expression in a panel of cDNA samples derived from the tumors described above. In human brain, the 7 kb transcript is highly expressed as shown in Fig. 4. RT-PCR assay detected all the major transcripts in normal brain samples. We, however, found that expression of some of the *Hqk* transcripts was altered in brain tumor samples (Fig. 4A). In six specimens out of 20 glioma samples, *Hqk* expression was clearly downregulated. For example, in samples No. 5, 17, and 20, the 7 kb transcript was specifically missing, and all three major transcripts were not detected in the samples, No. 8 and 19. Only the 5 kb transcript was downregulated in sample No. 18. In contrast, no alterations were found in three schwannomas and in eight meningioma samples (Fig. 4B). Northern blot analyses using RNAs from samples No. 2, 5, 8, 12, and 17 unambiguously showed that the 7 kb transcript was not expressed in samples No. 5, 8, and 17 (Fig. 5). On the other hand, a band of almost the same size as those in samples 2 and 12 was clearly observed in lane M, on which mouse brain RNA was loaded. This band in lane M represents the 7 kb message of *qkI*, as the 7 kb-B transcript is scarcely expressed in mouse brain.[2] The above data suggest that the *Hqk* messages, especially the 7 kb transcript, was downregulated in some of the glial tumors, but not in the tumors of other origins (Table III).

Table III. Summary of *Hqk* Expression in Human Brain Tumors

Tumor No.	Tumor type	Expression of *Hqk*-5		Expression of *Hqk*-6		Expression of *Hqk*-7	
		RT-PCR	Northern blot analysis	RT-PCR	Northern blot analysis	RT-PCR	Northern blot analysis
5	AA	+	+	+	ND	–	–
8	AA	–	–	–	ND	–	–
17	GBM	+	+	+	ND	–	–
18	GBM	–	ND	–	ND	–	ND
19	GBM	+	ND	+	ND	+	ND
20	GBM	+	ND	+	ND	–	ND
Normal		+	+	+	ND	+	+

+, normal level expression; –, no *Hqk* expression; ND, not determined.
AA: anaplastic astrocytoma. GBM: glioblastoma multiforme.

DISCUSSION

In this study, we described the isolation of *Hqk*, a human homolog of mouse *qkI* gene, and its genomic organization and expression pattern in human tissues. Comparison of the genomic structure of *Hqk* with that of *qkI* revealed remarkable similarities between the two genes. It is striking that there is no sequence differences at the amino acid level, and that there is 96% identity at the nucleotide level for the three major transcripts. Moreover, both 5′-UTR and 3′-UTR sequences are highly conserved; for example, parts of the 3′-UTR of *Hqk* are 85–97% homologous to that of mouse *qkI* (data not shown). Such a high sequence conservation suggests that this region may play an important role in the stability, localization, and translational control of the *Hqk* gene.

The *Hqk* gene consists of 8 exons distributed over a genomic region of approximately 200 kb. The exon sizes and the exon boundary locations are conserved between human and mouse. However, minor differences in the exon/intron organization were apparent. For example, comparison between the predicted *HQK7-B* and *QKI7-B* sequences[2] showed that the human protein is larger, with 4 additional amino acids at the carboxy-terminus (data not shown). Whether this particular isoform is translated in human tissues is not known at present. In the 5′ upstream region of the *Hqk* gene, we failed to find the intron corresponding to intron 1 of *qkI*.[2] Instead, a larger exon 1 which includes sequences corresponding to mouse exon 1, intron 1 and exon 2 is present in the *Hqk* gene. We obtained a 4 kb sequence upstream to the putative translation start site. Comparative analysis demonstrated high sequence similarity in this region, especially in the ~800 bp region upstream to the start codon (nearly 80%). Given this high similarity, it is possible that the 800 bp region might contain *cis*-element(s) required for proper *Hqk* expression or the initiation site for *Hqk* transcription. However, in the 4 kb genomic region containing the 800 bp sequence, no TATA or other sequences related to promoter functions could be detected, suggesting that the *Hqk* may be driven by a TATA-less promoter.[2]

Northern blot analysis and RT-PCR (data not shown) showed that *Hqk* messages are expressed not only in adult, but also in fetal heart and brain, suggesting possible roles in human development. We noticed that the *Hqk* expression pattern was different from that of mouse, though this might be due to differences in age or sex of individuals from which the RNA samples were derived. In human brain, the 7 kb transcript is more abundant than the 5 kb transcript, while the 5 kb message is predominant in heart. In mouse, similar levels of the three major isoforms were detected in brain. In addition, we found that the 7 kb transcript in testis is shorter, when the northern blot analysis was performed using the 7 kb specific probe, indicating another tissue-specific alternative transcription in this gene. Similarly, a band larger than 7 kb detected in heart (Fig. 3A) might represent an uncharacterized alternative transcript as this does not correspond to either the 7 kb or the 7 kb-B message.

Members of the *quaking* gene family are thought to play important roles in various biological processes such as development, cell differentiation and cell proliferation. Mutations in the *qkI* homolog, e.g. *gld-1* in nematode or *who/how* in fruit fly cause abnormal cell proliferation or cell migration. In the CNS of mice, reduction of *qkI* expression results in a hypomyelination phenotype.[4] Considering these facts, it is likely that alterations in the *Hqk* gene itself or its gene expression level may lead to disease conditions in humans. We have assigned the *Hqk* gene to the chromosomal region 6q25–26, to which no human neurological diseases have been mapped. However, a high incidence of chromosomal aberrations at 6q23.3 to 26 in astrocytic tumors has been reported.[35–37] Introduction of a fragment of normal chromosome 6 into a tumor cell line with 6q deletion resulted in suppression of tumorigenicity, suggesting the presence of tumor suppressor gene(s) on the corresponding chromosomal region.[38] On the other hand, a null mutation in the *gld-1* gene, a *C. elegans* homolog of *qkI*, leads to excessive oocyte growth, and partial loss-of-function mutation in *gld-1* causes oocytes to arrest at the pachytene stage.[13, 17] These results clearly suggest the involvement of the *gld-1* in the regulation of cell proliferation. More importantly, Pilotte *et al.*[33] recently reported that the *QKI-7* isoform could act as a potent apoptosis inducer *in vitro*. *QKI* protein and possibly *Hqk* gene product function as RNA-binding proteins. There is increasing evidence for the involvement of RNA-binding proteins in tumorigenesis. *MCG10*, a p53 target gene, encodes a protein containing two KH domains, which can suppress cell proliferation by inducing apoptosis and cell cycle arrest at G2–M. Thus, this KH domain RNA-binding protein is considered as a tumor suppressor.[39] On the other hand, a KH domain protein, *KOC*, was overexpressed in cancers.[32] *Musashi 1* containing two ribonucleoprotein (RNP)-type RNA-binding domains (RBDs) is also overexpressed in malignant gliomas.[40] These results suggest that various types of RNA-binding proteins are involved in cell proliferation, and expression alterations of those RNA-binding proteins often cause tumor formation. This prompted us to examine the expression of *Hqk* in human tumors. Since *Hqk-7* is highly expressed in brain, and *Hqk-7* is likely to be an apoptosis inducer,[33] we suspected that loss or reduction of *Hqk-7* expression would be detected in brain tumor samples. Our results indeed demonstrated a high incidence of expression alterations of *Hqk* in gliomas (30%; 6/20), but not in other tumors such as schwannomas (0/3), or meningiomas (0/8). Among the tumor samples showing expression alter-

ations, two were devoid of all three major transcripts, one was missing only the 5 kb message, and only the 7 kb message was absent in the last two samples (Fig. 4). Studies on mouse *qkI* genomic structure[2] suggest that three major alternative messages are transcribed from a single transcription initiation site, and that the expression of each isoform is regulated at the level of splicing. Thus, genetic or epigenetic changes in the promoter region would result in loss of the three alternative transcripts. Specific loss of one of the alternative messages can be caused by alterations in the sequences required for proper splicing. Our examinations of genomic DNA from the glioma patients failed to detect apparent genetic DNA rearrangements or point mutations in coding sequences or splicing donor/acceptor sequences. However, epigenetic changes associated with tumorigenesis may cause silencing in *Hqk* gene expression[41] or alterations in splicing regulation. In any case, frequent loss of *Hqk-7* in high-grade glioma (Figs. 4, 5) may suggest that this expression alteration is directly related to malignant glioma formation. Recent findings by Pilotte *et al.*[33] lend support this notion. The authors reported that among three major *qkI* isoforms, only *QKI-7* could induce apoptosis *in vitro* and that life or death of *qkI*-expressing cells would be determined by the balance between the *QKI* isoforms generated by alternative splicing. The authors claim that *QKI-7* is a sufficient apoptotic inducer if present in cytoplasm, while nuclear translocation of *QKI-7* suppresses its apoptosis-inducing ability. *QKI-5* predominantly localized in nucleus can form heterodimers with *QKI-7*[33] and could cause the nuclear translocation of *QKI-7*, resulting in cell survival. The relative level of each isoform would be crucial for cell death/proliferation switching. It is thus possible that not only the total loss of *Hqk* transcripts, but also alterations in the balance between the isoform levels discovered in this study may result in progression of glial cell tumors. It is important to screen *Hqk* gene abnormalities in glioma samples on a larger scale, and to elucidate their precise roles in tumorigenesis using mouse models. Isoform-specific knockout or conditional disruption of the *qkI* gene only in glial cells should yield information on the possible role of the *quaking* gene in tumor formation.

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