Editing of hnRNP K protein mRNA in colorectal adenocarcinoma and surrounding mucosa

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The heterogeneous nuclear ribonucleoprotein K (hnRNP K) protein is an RNA-binding protein involved in many processes that compose gene expression. K protein is upregulated in the malignant processes and has been shown to modulate the expression of genes involved in mitogenic responses and tumorigenesis. To explore the possibility that there are alternative isoforms of K protein expressed in colon cancer, we amplified and sequenced K protein mRNA that was isolated from colorectal cancers as well as from normal tissues surrounding the tumours. Sequencing revealed a single G-to-A base substitution at position 274 that was found in tumours and surrounding mucosa, but not in individuals that had no colorectal tumour. This substitution most likely reflects an RNA editing event because it was not found in the corresponding genomic DNAs. Sequencing of RNA from normal colonic mucosa of patients with prior resection of colorectal cancer revealed only the wild-type K protein transcript, indicating that G274A isoform is tumour related. To our knowledge, this is the first example of an RNA editing event in cancer and its surrounding tissue, a finding that may offer a new diagnostic and treatment marker.

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Colorectal cancer is one of the most frequent causes of morbidity and mortality from malignancy in Europe and North America (Didkowska et al, 2003; Jemal et al, 2005). Although improvements have been made in the surgical and nonsurgical therapies of colorectal cancer, the mortality remains high. A better understanding of the molecular basis of the disease is needed to develop new diagnostic tools and improve ways to prevent and treat colorectal cancer.

The heterogeneous nuclear ribonucleoprotein K (hnRNP K) protein is an ancient RNA-binding protein (Bomsztyk et al, 2004). Since it was originally identified as a component of the heterogeneous nuclear ribonucleoprotein particles, K protein has been also found in the cytoplasm and mitochondria where it is involved in multiple processes that compose gene expression (Bomsztyk et al, 2004). K protein contains three K homology (KH) domains that mediate RNA/DNA binding and the K-interactive (KI) region that recruits many protein partners (Bomsztyk et al, 1997, 2004).

K protein is upregulated in malignant cells (Dejgaard et al, 1994; Mandal et al, 2001; Ostrowski and Bomsztyk, 2003; Li et al, 2004), and it regulates the expression of genes involved in mitogenic responses, indicating that it has a direct role in cancerogenesis (Micheletti et al, 1995, 1996; Ostrowski and Bomsztyk, 2003; Ostrowski et al, 2004). We have shown that following mitogenic stimulation, K protein is recruited to gene loci known to be upregulated in malignant states, including c-myc (Ostrowski et al, 2003). This finding is consistent with the observed shift of K protein to the nuclei in neoplasms (Ostrowski and Bomsztyk, 2003).

As a result of alternative splicing, there are several known isoforms of K protein (Dejgaard et al, 1994; Makeyev and Liebhaber, 2002). To explore the possibility that there are isoforms of K protein enriched in cancer, we sequenced cDNA encoding K protein from colorectal adenocarcinoma and compared it to the cDNAs from the surrounding normal mucosa. Sequencing revealed novel G274A polymorphism found in mRNA, but not in the genomic DNA. Thus, this polymorphism most likely reflects mRNA editing event, which changes alanine (Ala) to threonine (Thr) at the end of KH1 domain. Beside colorectal adenocarcinoma, G274A isoform of K protein mRNA was also found in a few thyroid cancers, but not in normal tissues surrounding breast, thyroid or renal carcinomas, nor in normal or in inflammation-altered colonic mucosa from the patients with inflammatory bowel disorders. These data identify novel RNA editing phenomenon specific to colorectal carcinoma that may be involved in the processes of tumour formation and development and may serve as a diagnostic marker.

MATERIALS AND METHODS

Tissues

In all, 21 surgically resected colorectal tumours, 18 thyroid tumours (five follicular, four papillary and nine medullary
was purified with QIAquick PCR Purification Kit (Qiagen), cDNA inserted into plasmid vector was amplified, the PCR product creates ScaI system (Applied Biosystems, Foster City, CA, USA). The polymerase chain reaction (PCR)-amplified DNA fragments were sequenced using ABI Prism 377 automated DNA sequencing. DNA was isolated from randomly chosen bacterial clones and were transformed into chemically competent TOP10 cells. Plasmid DNA was purified by affinity chromatography using Ni-NTA agarose (Qiagen) according to the manufacturer’s protocol. The correct point-mutant product was confirmed by sequence analysis using liquid chromatography–electrospray mass spectrometry with collisional fragmentation (LC–ESI–MS–MS/MS).

Purified wild-type and mutated K proteins were phosphorylated in vitro by casein kinase-1 (CK1), casein kinase-2 (CK2) or their mixture in the buffer containing 25 mM Tris, pH = 7.5, 200 mM NaCl, 0.1 mM ATP (with or without [γ-32P]-ATP) and 10 mM MgCl2 for 20 min at 30°C. Proteins phosphorylated in the presence of [γ-32P]-ATP were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and visualised using a phosphorimager. Proteins phosphorylated with cold ATP were digested with trypsin, and the resulting phosphopeptides were analysed by mass spectrometry. Peptide mixture was applied to nano-high-performance liquid chromatography RP-18 column, directly coupled to nano-Z-spray ion source of quadrapole time-of-flight electrospray–mass spectrometry (Waters) working in the regimen of data-dependent MS to MS/MS. Proteins phosphorylated by cold ATP were digested with trypsin, and the resulting phosphopeptides were analysed by mass spectrometry. Peptide mixture was applied to nano-high-performance liquid chromatography RP-18 column, directly coupled to nano-Z-spray ion source of quadrapole time-of-flight electrospray–mass spectrometry (Waters) working in the regimen of data-dependent MS to MS/MS. The output list of parent and daughter ions was submitted to database search using MASCOT (MatrixScience) program. Peptide identification and the presence of their covalent modifications were verified by inspection of parent mass fragmentation patterns using programs MassLynx (Waters) and ProteinProspector.

### Protein modelling

The three-dimensional (3D) structures of the KH1 domain of protein K and its mutant A92T, and of the KH2 domain were predicted by homology modelling using SWISS-MODEL (Peitsch, 1996), using the coordinates of the KH3 domain as the template (crystallographic structure 1 khm in Protein Data Bank). The structure of the entire K protein was modelled by joining the structures of the individual domains by the linker in an extended conformation. The preliminary model of KH1–RNA complex was obtained by copying the coordinates of the RNA molecule from the KH3–RNA structure obtained by NMR (Bradock et al, 2002) and optimising the interactions with the protein by the steepest descent
method, as implemented in HyperChem 7.1 (Hypercube Inc., Gainesville FL, USA).

Putative phosphorylation sites in K protein were predicted using the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al, 1999). The 3D model of protein K was used to identify those potential phosphorylation sites, which are not buried in the protein core, and thus are likely to be modified in the native protein.

Statistical analysis Differences between groups were analysed by the Mann–Whitney test and paired two-tailed t-test using Statistica PL software. A P-value of <0.05 was considered to be statistically significant.

RESULTS

Pilot sequencing of the full-length cDNA encoding K protein isolated from three pairs of colon adenocarcinoma and the normal mucosa surrounding the tumour revealed G274A polymorphism present in all samples (Accession number AY911506). To further explore this finding, cDNAs were generated from RNA isolated from several pairs of tissues (normal/pathological) that were taken from patients with colorectal cancer. A 453-bp fragment of K protein cDNA was amplified by PCR, T/A subcloned into plasmid vector and six to 14 bacterial clones from each transformation were randomly chosen for sequencing (Figure 2).

As shown in Table 2, G274A polymorphism was found in K protein cDNA generated from RNA that was isolated from 15 out of 21 (71%) colorectal cancers and from all normal mucosa that surrounded these colon tumours. The median percentage of K protein transcripts carrying G274A polymorphism within an individual tissue specimen were significantly higher in normal mucosa surrounding tumours than in corresponding colorectal carcinomas (54 vs 8% of all sequenced clones; P<0.001). One explanation for such a difference could be that tumour induces G274A RNA editing. Alternatively, normal mucosa in response to tumour may produce a signal for the RNA editing. The relative frequency of the clones with G274A polymorphism did not appear to correlate with any of the variables that were tested, such as sex, age, tumour location, size, type, differentiation, presence of metastases and stage according to Astler–Coller, Jass prognostic criteria. This observation suggests that G274A polymorphism is a good candidate to be used as a universal and specific marker for the diagnosis of colorectal adenocarcinoma.

The above studies suggested that the G274A polymorphism is related to colorectal adenocarcinoma. To test this relationship further, we sequenced K protein cDNA from normal mucosa taken

![Figure 2](image_url)

**Figure 2** Analysis of K protein transcript. (A) Sequences of individually cloned cDNA fragments from wild-type and edited mRNA of K protein. ScaI restriction site is indicated by box. (B) Agarose gel electrophoresis of PCR-generated fragments from cDNA encoding K protein that were digested with (right) or without (left) ScaI restriction enzyme. Fragments (453 bp) of K protein cDNA wild type (line 1) and with G274A polymorphism (line 2) were digested with ScaI.

| Patient groups                      | Number of tissue pairs | Number of tissues with G274A RNA isoform | Number of sequenced clones: total (G274A RNA) |
|-------------------------------------|------------------------|-----------------------------------------|-----------------------------------------------|
|                                     |                        | Normal tissue/pathological tissue       | Normal tissue/pathological tissue             |
| Colon cancer                        | 21                     | 21/15                                   | 229 (120)/235 (51)                            |
| Colon cancer after surgical treatment| 10                     | 0/NA                                    | 80 (0)/NA                                    |
| Crohn’s disease or ulcerative colitis| 18                     | 0/0                                     | 54 (0)/54 (0)                                |
| Thyroid cancer                      | 9                      | 0/0                                     | 108 (0)/108 (5)                              |
| Breast cancer                       | 20                     | 0/0                                     | 120 (0)/120 (0)                              |
| Renal cancer                        | 10                     | 0/0                                     | 80 (0)/80 (0)                                |

NA = not applicable.
colonoscopy from the sites of surgical anastomosis from 10 patients with prior resection of colorectal carcinoma, including two patients whose tissues were previously tested positive for the G274-A polymorphism in both cancer and surrounding mucosa. In all those patients, only the G274 wild-type isoform was found. Similarly, in nine patients with inflammatory bowel disease, sequencing analysis of biopsy specimens of the involved and unaffected mucosa revealed only the wild-type K protein mRNA. To test if the G274A polymorphism is specific to colorectal adenocarcinoma, we examined pairs of neoplastic and normal surrounding tissues from breast, kidney and thyroid. Except for two thyroid cancers, only the wild-genotype K protein RNA isoform was found in these specimens (Table 2). The fact that the G274A polymorphism was also found in thyroid cancers suggests that the RNA editing could be also induced by other tumours, although with much lower efficiency.

To exclude the possibility that the G274A polymorphism was generated by reverse transcriptase (RT) – PCR, we repeated the entire genotyping experiment in all colorectal patients. The second series of RNA extraction and sequencing confirmed the presence of previously determined polymorphism in both normal and cancer samples (data not shown). This indicates that G274A polymorphism was not introduced by PCR.

Next, we tested if G274A polymorphism is found in K protein gene. To do that, we analysed 78 samples of genomic DNA (Figure 1) obtained from tumour and matched surrounding normal tissue samples extracted from the same 21 patients with colorectal cancer and 18 patients with thyroid cancer. The PCR products were subcloned into plasmid vector, and five clones from each bacterial transformation were sequenced. Of 390 independent products were subcloned into plasmid vector, and five clones from each bacterial transformation were sequenced. Of 390 independent clones analysed, none contained the G274A polymorphism. Since neither CK1 nor CK2 were digested with trypsin. The resulting peptides were analysed by LC–ESI–MS–MS/MS mass spectrometry. Amino-acid sequencing identified the 87- to 103-amino-acid wild-type K protein peptide (ILSISAVKKEI) (bold T indicates substituted Thr at position 92) and the mutated peptide (ILSISAVKKEIK) (bold A indicates Ala at position 92) and the mutated peptide (ILSISAVKKEIK) (bold A indicates Ala at position 92) and the mutated peptide (ILSISAVKKEIK) (bold A indicates Ala at position 92) and the mutated peptide (ILSISAVKKEIK) (bold A indicates Ala at position 92) and the mutated peptide (ILSISAVKKEIK) (bold A indicates Ala at position 92) and the mutated peptide (ILSISAVKKEIK) (bold T indicates substituted Thr at position 92) molecules with the masses 1843.07 and 1873.08 Da, respectively, as well as with 80 and 160 Da above the predicted masses. The G274A polymorphism found in K protein mRNA substitutes Ala codon by Thr codon. One consequence of such amino-acid substitution within K protein could be a creation of new phosphorylation site. Reversible phosphorylation of proteins is a fundamental mechanism of the regulation of cellular functions. Analysis of K protein amino-acid sequence revealed several putative sites that meet the criteria for phosphorylation by serine–threonine kinases: calmodulin CII, CK1, CK2, glycogen synthase kinase 3, p34cdc2, p70S6 and protein kinase C (PKC).

Indeed, K protein has been shown to be phosphorylated by several kinases, including Src, Lck, PKCβ, extracellular signal-regulated protein kinase, c-Jun NH2-terminal kinase and CK2, each phosphorylating one or more sites (Bomszytk et al., 2004). Three-dimensional modelling of K protein indicates that Thr92, introduced as a result of G274A editing of mRNA, is located on the surface of the molecule (Figure 3; panels A–C), and thus may represent a new phosphorylation site for CK1. We tested this possibility next.

To prove that increased phosphorylation of mutated K protein reflects phosphorylation of Thr92, we used mass spectrometry analysis. The wild-type and mutated proteins phosphorylated by both CK1 and CK2 were digested with trypsin. The resulting peptides were analysed by LC–ESI–MS–MS/MS mass spectrometry. Amino-acid sequencing identified the 87- to 103-amino-acid wild-type K protein peptide (ILSISADTTIEGILKK) (bold A indicates Ala at position 92) and the mutated peptide (ILSISTDIE-TIGELKKK) (bold T indicates substituted Thr at position 92) molecules with the masses 1843.07 and 1873.08 Da, respectively, as well as with 80 and 160 Da above the predicted masses. The
addition of a phosphate group increases the mass of peptides by 80 Da. Thus, as shown in Table 3, the A92T isomorph was much more efficiently phosphorylated by these kinases, an alteration that most likely reflects direct phosphorylation of Threonine 92.

In the wild-type peptide, there are three potential phosphorylation sites adjacent to Ala92, Ser89, Ser91 and Thr96, but only Thr96 fits the criteria for phosphorylation by CK2. Thus, the characteristic mass shift of 0.7% of the wild-type peptide molecules likely reflects P-Thr96. The Thr92 residue, introduced by RNA editing, also meets the criteria for phosphorylation by either CK1 or CK2. Therefore, 22.5% of mutated peptide molecules with added 80 Da might reflect P-Thr92, and other 2.5% of phosphopeptides are consistent with both P-Thr92 and P-Thr96. Thus, MS studies provide evidence that Thr92 residue is either itself phosphorylated or enhances phosphorylation of the neighbouring Ser/Thr residues.

K protein binding to RNA reflects synergistic interaction between three KH domains and cognate RNA where each KH domain binds one of three C-rich boxes (Paziewska et al, 2004). As shown in Figure 3, the structural changes resulting from the putative phosphorylation of Thr92 does not disrupt the 3D-fold of the KH domain (panels A–C). However, the phosphorylation can introduce significant negative charge on the protein surface (panels D–F), leading to alteration of K protein RNA-binding activity. Independent of whether Thr92 is directly phosphorylated or changes phosphorylation of other neighbouring residues, these observations indicate that A92T polymorphism may alter K protein function in response to extracellular signals that engage kinase cascades. The putative location of the RNA molecule (panels G–I) (based on solved structure of the KH3 single-stranded DNA complex; Braddock et al, 2002) is shown to visualise the region of protein–RNA interactions that may be influenced by the Ala to Thr substitution and by the phosphorylation. Although not shown in this figure, because of the lack of the appropriate modelling template, the extended 3′-end of the RNA molecule could directly interact with the modified region.

**DISCUSSION**

RNA editing is a co- or post-transcriptional process (Gott and Emeson, 2000) that modifies the genetic information at the transcript level, either by insertion or deletion of nucleotides, or by base modification. Enzymatic base modification of RNA sequences was originally discovered in the mitochondrial RNA of trypanosomes (Turelli and Trono, 2005). In mammals, the two major types of RNA base modifications are associated with various physiological processes, including neurotransmission, lipid metabolism and adaptive immunity (Turelli and Trono, 2005). They include A-to-I and C-to-U editing (Gott and Emeson, 2000; Maas and Rich, 2000; Davidson, 2002).

RNA editing is mediated by RNA–protein complexes, which contain small guiding RNAs and several associated proteins (Decatur and Fournier, 2003). A-to-I RNA editing occurs on unspliced mRNA templates and is mediated by adenosine deaminases acting on double-stranded RNAs. The partially double-stranded RNA that involves exonic and intronic sequences or a hairpin structure is thought to be essential for this type of RNA editing (Maas et al, 2003). The primate cytidine deaminase family represents 12 enzymes (Turelli and Trono, 2005). Adenosine deaminases acting on double-stranded RNAs are responsible for site-specific RNA editing of both coding and noncoding region sequences (Yang et al, 2005). Adenosine deaminases acting on double-stranded RNA-mediated RNA editing occurs within coding or untranslated RNAs and appears to be widespread in human nervous system (Blow et al, 2004; Blow et al, 2004). Adenosine deaminases acting on double-stranded RNA-mediated RNA editing is essential for normal development and cell viability, and altered editing is associated with inflammation and several neurological disorders (Geslain and Ribas de Pouplana, 2004; Eisenberg et al, 2005).

Until now, a few C-to-U edits and single examples of purine-to-pyrimidine and pyrimidine-to-purine RNA edits were described within the open-reading frame of cellular and viral transcripts (Gott and Emeson, 2000; Maas and Rich, 2000; Gerber and Keller,

![Figure 4](image-url) **Figure 4** Phosphorylation of K protein and its mutant by casein kinases (CK). Recombinant wild-type and Thr92-mutated K proteins were phosphorylated using CK1 (CK1, rat, recombinant) or CK2 (CKII purified from rat liver) or both kinases. 32P-labelled proteins eluted from the beads are separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis followed by autoradiography (upper panel) and densitometry of the Coomassie-stained band of the same gel (lower panel) using Molecular Imager FX Pro Plus (Bio-Rad). The 32P signals were normalised to Coomassie band intensities and expressed as relative levels (diagram). Results shown in the graph represent means ± s.d. of three independent experiments.

| Table 3 | Summary of peptide (87–103 aa) counts derived from the wild-type and A92T-mutated K protein that were phosphorylated by CK1 and CK2 in vitro and analysed by mass spectrometry |
|---------|---------------------------------------------------------------------------------|
|          | ILSISADYTEGIEILKK mass 1843.07 Da (% of total) | ILSISTDYTEGIEILKK mass 1873.08 Da (% of total) |
| Total peptide counts* | 1569 (100) | 3360 (100) |
| Number of peptide molecules with the mass 80 Da above predicted | 10 (0.7) | 775 (22.5) |
| Number of peptide molecules with the mass 160 Da above predicted | ND | 86 (2.5) |

aa = amino acids; CK1 = casein kinase 1; CK2 = casein kinase 2; ND = not detected. *Total peptide counts is the number of detected peptide molecules with the predicted mass as well as with the masses 80 and 160 Da above predicted. Itallic A or T in peptides sequences indicates Ala or substituted Thr at position 92, respectively.

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Figure 5 mRNA editing of G to A generates a novel K protein Thr92 isoform. (A) The G to A base substitution at 274 in K protein transcript changes Ala92 to Thr92 located at the end of the KH1 domain. KH domains are shown in green. (B) K protein RNA sequence analysis around the editing site reveals a hairpin conformation.

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