

Frequent alterations of the PI3K/AKT/mTOR pathways in hereditary nonpolyposis colorectal cancer

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Abstract The phosphatidylinositol 3-kinases-AKT-mammalian target of rapamycin pathway (PI3K/AKT/mTOR) is central in colorectal tumors. Data on its role in hereditary cancers are, however, scarce and we therefore characterized mutations in *PIK3CA* and *KRAS*, and expression of PIK3CA, phosphorylated AKT, and PTEN in colorectal cancers linked to hereditary nonpolyposis colorectal cancer (HNPCC). Sequencing was used to identify mutations in *PIK3CA*, a real-time PCR-based method to identify *KRAS* mutations, and immunohistochemical staining was used to evaluate the expression of PIK3CA, phosphorylated AKT and PTEN in 58 HNPCC-associated colorectal cancers. Derangements of at least one of the PI3K/AKT/mTOR components analyzed were found in 51/58 (88%) tumors. Mutations in *PIK3CA* and *KRAS* were identified in 14 and 31% of the tumors respectively. Overexpression of PIK3CA and phosphorylated AKT occurred in 59 and 75% and were strongly associated ($P = 0.005$). Reduced/lost PTEN expression was found in 63% of the tumors. Though HNPCC-associated colorectal cancers show simple genetic profiles with few chromosomal alterations, we demonstrate frequent and repeated targeting of the PI3K/AKT/mTOR

pathway, which suggests that therapeutic strategies directed at this pathway are likely to be beneficial also in HNPCC.

Keywords Hereditary nonpolyposis colorectal cancer (HNPCC) · *KRAS* · Microsatellite instability · PI3K/AKT/mTOR · PTEN

Introduction

Key components of the phosphatidylinositol 3-kinases-AKT-mammalian target of rapamycin pathway (PI3K/AKT/mTOR) are common targets for aberrant signaling in solid tumors [1]. PI3K increases the intracellular concentration of phosphatidylinositol-3,4,5-triphosphate (PIP₃), which acts as a second messenger in the activation of downstream pathways. Activation of AKT, regulated by PTEN, increases cell proliferation, adherence, transformation and survival [2, 3]. *PIK3CA* (p110 α) encodes for the catalytic subunit of PI3K, and somatic mutations have been demonstrated in several cancer types, including 10–32% of colon cancers [4–7]. Since derangements of the PI3K/AKT/mTOR pathway influence migration and invasion, key molecules in this pathway represent potential therapeutic targets and may through interaction with the RAS-RAF pathway also predict response to anti-EGFR therapies [8].

Colorectal cancer develops through alterations in distinct molecular pathways, characterized by chromosomal instability (CIN), microsatellite instability (MSI) and the more recently discovered CpG island methylator phenotype (CIMP) [9]. Hereditary nonpolyposis colorectal cancer (HNPCC) accounts for 1–4% of colorectal cancer and is characterized by tumor development at young age, a predilection for mucinous and poorly differentiated tumors

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and a tumor location preferably in the proximal colon [10]. HNPCC has been linked to germline mismatch repair (MMR) gene mutations. The MMR defect leads to widespread MSI and a phenotype characterized by few gross genetic alterations [11–13]. It has therefore generally been accepted that mutations in coding repeats in tumor suppressors drive MSI tumorigenesis [14]. The contribution of pathways linked to sporadic colorectal cancer development and progression is, however, less understood. Since the PI3K/AKT/mTOR pathway is central in colorectal tumorigenesis and represents a promising therapeutic target, we aimed to investigate its role in HNPCC associated colorectal cancer.

Materials and methods

Patient materials

Fifty eight HNPCC-associated colorectal cancers from 55 patients with disease-predisposing MMR gene defects in *MSH2* ($n = 35$), *MLH1* ($n = 16$), or *MSH6* ($n = 4$) were included in the analysis. The mean age was 46 (23–78) years and the series included 25 females and 30 males. Tumor location was within the colon in 47 cases and the rectum in 11. All patients had undergone genetic counseling at Lund University hospital and the study was approved by the ethics committee at Lund University.

DNA isolation and PIK3CA and KRAS mutation screening

DNA was extracted from $3 \times 10\text{-}\mu\text{m}$ sections of formalin-fixed paraffin-embedded tissue ($n = 52$) or from microdissected frozen tissue ($n = 6$) according to standard procedures [15]. Exons 9 and 20 of *PIK3CA* were amplified and sequenced using the Terminator Cycle Sequencing Reaction Kits version 3.1 and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA) as previously described [16]. *KRAS* mutations were identified using the DxS real-time PCR based kit (Roche Diagnostics, Basel, Switzerland), which detects seven different mutations in codon 12 and 13 of *KRAS*.

Immunohistochemistry of PIK3CA, phospho-Akt and PTEN

Immunohistochemical stainings were performed using $4\text{-}\mu\text{m}$ sections of formalin-fixed, paraffin embedded tissue, which were mounted on DAKO ChemMate Capillary Gap Microscope Slides (DAKO A/S, Glostrup, Denmark) and dried at room temperature overnight followed by incubation at 57°C for 2 h. The tissue sections were

deparaffinized in xylol and rehydrated through descending concentrations of alcohol. Antigen retrieval was achieved by micro-wave treatment in 1 mM EDTA at 800 W for 8 min followed by 15 min at 300 W. The slides were then allowed to cool for at least 20 min in EDTA solution. Immunohistochemical staining was performed in an automated immunostainer (TechMate 500 Plus, DAKO), according to the manufacturers instructions with a 2 h incubation of the antibodies.

The PIK3CA antibody (#4254, PI3 kinase p110 α antibody, diluted 1:50, Cell Signaling Technology, Beverly, MA, USA) detects endogenous levels of the p110 α subunit of the PI3K protein whereas the phospho-AKT antibody (#3787 Phospho-AKT, diluted 1:50, Cell Signaling Technology) detects AKT1 only when phosphorylated at serine 473 and AKT2 and AKT3 only when phosphorylated at equivalent sites. Scoring of immunohistochemical staining was independently done by A. E. and M. N. For PIK3CA and phospho-AKT staining, a total score was generated based on the number of stained tumor cells and the staining intensity as described [17]. The percentage of stained tumor cells was graded as 0 (no stained tumor cells), 1 (1–25%), 2 (26–50%) and 3 (51–100%) and the intensity of the staining was classified as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Tumors with a total score of 0–4 were considered to have low expression and tumors with a score ≥ 5 high expression. Normal epithelial and stromal cells served as internal controls in the calculation of the intensity and extent of staining. PTEN expression (#9559 PTEN (138G6), diluted 1:100, Cell Signaling Technology) was evaluated as downregulated or positive relative to the staining of the internal control in each section.

Statistical analysis

Statistical analysis was performed using STATA and applied Fischers's exact test (StataCorp. 2005. Stata Statistical Software: Release 9. College Station, TX: StataCorp LP).

Results

Mutation analysis

PIK3CA mutations were identified in 5/36 (14%) tumors and included the missense mutations p.E454K, p.E545G, p.H1047R and the nonsense mutation p.W552X (Table 1). Three of the mutations, including the nonsense mutation, were found in the helical domain (exon 9) and one mutation was located in the kinase domain (exon 20) of *PIK3CA*. The mutations occurred in two colon cancers and

three rectal cancers. *KRAS* mutations were found in 31% (15/49) of the tumors, including 11 colon cancers and 4 rectal cancers. The most common mutations were p.G12D ($n = 9$), followed by p.G13D ($n = 6$) and p.G12V ($n = 1$) with coexisting *KRAS* mutations (p.G12D and p.G13D) in

one colon cancer (Table 1). Concurrent mutations of *PIK3CA* and *KRAS* were found in three tumors (11%).

Expression of PI3K pathway genes

Downregulation of PTEN, which acts as a negative regulator of PI3K signaling, was found in 31/49 (63%) of the tumors with total loss of expression in 11 cases (Fig. 1). All tumors with *PIK3CA* mutations showed downregulated PTEN expression. Increased *PIK3CA* expression was found in 29/49 (59%) tumors, including two of the four mutant tumors. Oncogenic *PIK3CA* stimulates downstream AKT-signaling and expression of phospho-AKT was classified as strong in 36/48 (75%) of the tumors. Expression of *PIK3CA* and phospho-AKT were strongly associated ($P = 0.005$, Fischer's exact t -test) with consistent changes in 33/44 (75%) tumors and concurrent upregulation in 55%.

Table 1 *PIK3CA* and *KRAS* mutations identified

| Gene | Mutation | Amino acid change | No. of tumors |
|---------------|----------|-------------------|---------------|
| <i>PIK3CA</i> | c.G1633A | p.E545K | 1 |
| <i>PIK3CA</i> | c.A1634G | p.E545G | 2 |
| <i>PIK3CA</i> | c.G1656A | p.W552X | 1 |
| <i>PIK3CA</i> | c.A3140G | p.H1047R | 1 |
| <i>KRAS</i> | c.G35A | p.G12D | 9 |
| <i>KRAS</i> | c.G35T | p.G12V | 1 |
| <i>KRAS</i> | c.G38A | p.G13D | 6 |

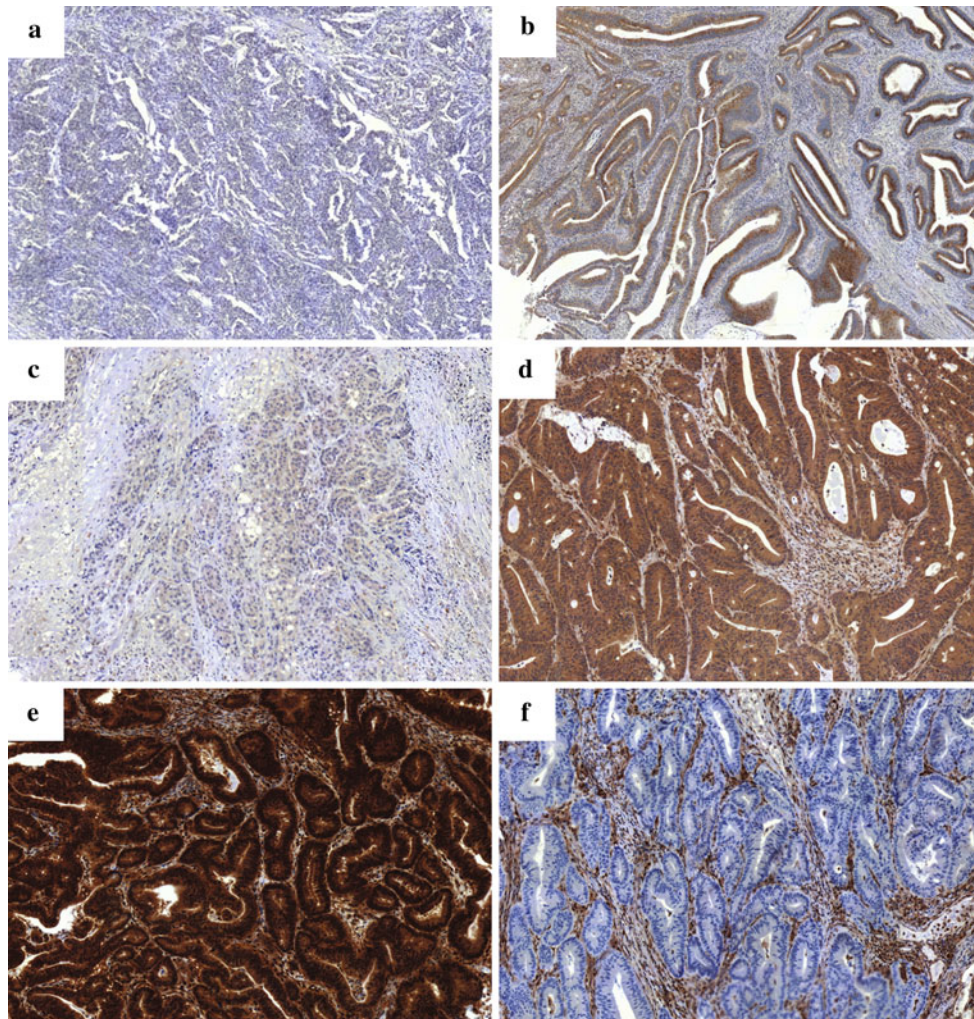


Fig. 1 Representative pictures demonstrating normal (a, c) and increased (b, d) expression of *PIK3CA* and phospho-AKT respectively. Retained PTEN expression is exemplified in (e) and lost PTEN expression in (f)

Discussion

The PI3K/AKT/mTOR signaling pathway is central in colorectal tumor development and harbors key targets for therapeutic intervention. Though HNPCC-associated tumors develop through defective MMR and display few karyotypic aberrations, PI3K/AKT/mTOR pathway genes show frequent alterations with 88% of the tumors showing alterations in at least one of the pathway components PIK3CA, phospho-AKT, KRAS and PTEN (Fig. 2). Our results support the findings in the single study that has previously demonstrated frequent alterations of PI3K/AKT/mTOR signaling in hereditary colon cancer [5]. We found a somewhat higher frequency of alterations (88 vs. 58%), which was attributed to more frequent overexpression (59%) of PIK3CA and downregulation (63%) of PTEN herein.

The frequency of *PIK3CA* mutations (14%) and *KRAS* mutations (31%) are comparable to the findings in sporadic colorectal cancer, albeit with a somewhat lower frequency of *KRAS* mutations than the 40% described in sporadic colorectal cancer (www.sanger.ac.uk/genetics/CGP/cosmic)

[5, 17, 18]. Several tumors had coexisting alterations in the PI3K/AKT/mTOR pathway, exemplified by a tumor carrying double *KRAS* mutations, a *PIK3CA* mutation, and deregulated expression of PIK3CA, phospho-AKT and PTEN. Repeated targeting of the PI3K/AKT/mTOR-pathway has also been recognized in cancers of the breast, colon and endometrium, and thus seems to confer an additive effect [16, 18, 19]. All tumors with *PIK3CA* mutations also showed decreased PTEN expression. This could be due to interaction of non-overlapping pathways and thereby circumvention of negative feedback [20]. Oncogenic *PIK3CA* mutations lead to increased downstream phosphorylation of AKT [21, 22]. Consequently, a strong association between the expression of PIK3CA and phospho-AKT was identified ($P = 0.005$, Fischer's exact test), and has been observed in other tumor types, e.g. squamous cell carcinoma of the head and neck and ovarian carcinoma [23, 24]. In contrast to protein expression level, *PIK3CA* mutations did not correlate with increased phospho-AKT expression, which may be explained by transcriptional or posttranscriptional mechanisms that determine the expression level of the catalytic subunit of PIK3CA. The strong association between

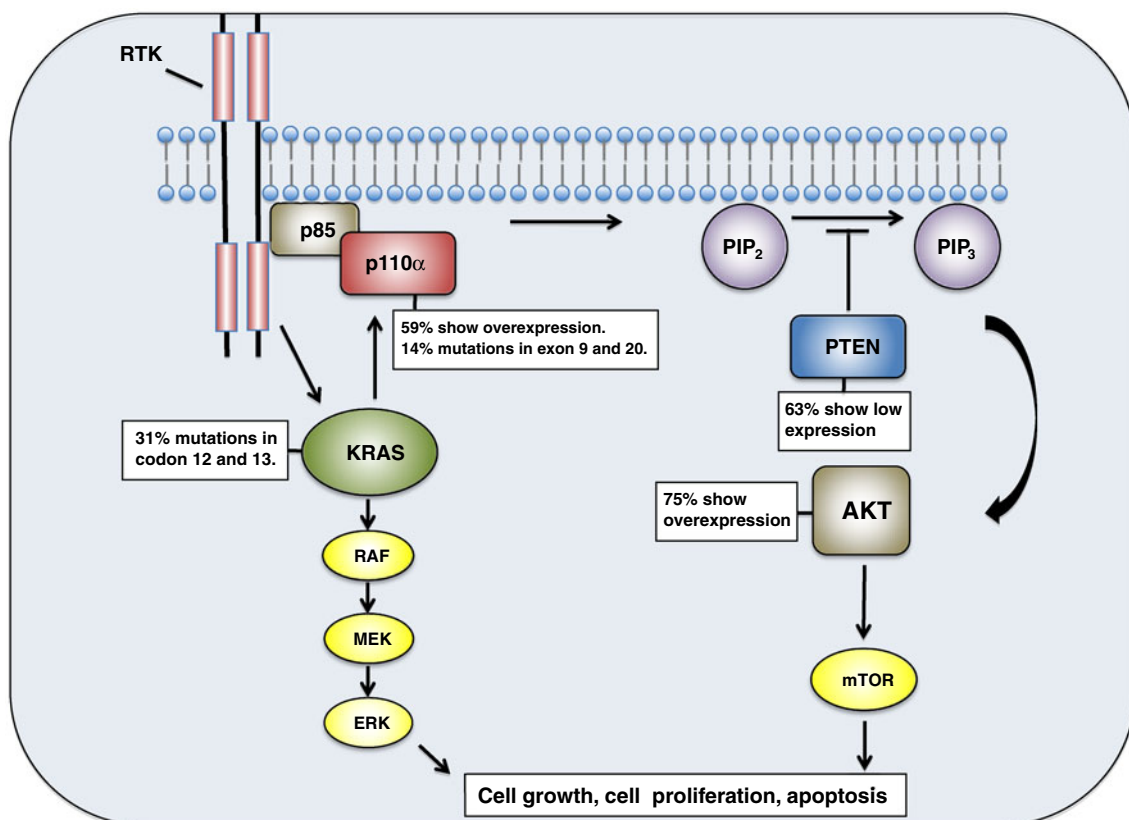


Fig. 2 Simplified model of the PI3K/AKT/mTOR signaling pathway. Growth factor binding to a tyrosine kinase receptor (RTK), activates the PI3K-complex (p110 α /p85). The lipid second messenger phosphatidylinositol (4,5) biphosphate (PIP₂) is converted by p110 α into phosphatidylinositol (3,4,5) triphosphate (PIP₃), a process regulated

by PTEN. AKT is thereby phosphorylated and hence, through downstream processes regulate cell growth, proliferation and apoptosis. The frequency of *PIK3CA* and *KRAS* mutations and protein expression alterations of PIK3CA, PTEN and AKT are demonstrated in the model

PIK3CA expression and phospho-AKT protein expression strongly indicates that AKT is the main downstream effector gene of PI3K and suggests PI3K-dependent AKT activation in HNPCC. The oncogenic effect of *PIK3CA* mutations have been demonstrated in both in vitro and in vivo studies [21, 22, 25–27] although the impact of the truncating mutation (p.W552X) in one of the rectal tumors is unclear since truncating mutations predominantly target tumor suppressors.

The PI3K/AKT/mTOR pathway has rapidly evolved to be a promising target in future therapy. In vitro studies support an antiproliferative and cytotoxic effect from PI3K/AKT/mTOR inhibition and recent data combining gene expression profiling and functional validation has indeed suggested that MSI colorectal cancer, whether arising from germline mutations in HNPCC or from somatic hypermethylation in sporadic cases, may indeed be particularly sensitive to compounds that inhibit the PI3K/AKT/mTOR pathways [4–7, 28].

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