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Immunohistochemistry is not an accurate first step towards the molecular diagnosis of *MUTYH*-associated polyposis

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Abstract Identifying patients with germline *MUTYH* mutation-associated polyposis is presently difficult. The aim of this study is to investigate the possibilities of IHC as a screening test to select patients for *MUTYH* mutation analysis. The expression of *MUTYH* protein in colorectal adenomas or cancer was studied by IHC using three different (1 polyclonal and 2 monoclonal) antibodies in six samples from patients with biallelic *MUTYH* mutations, in three samples from patients with a single *MUTYH* mutation, and in 11 samples from patients without *MUTYH* mutations. With the polyclonal antibody, adenomas and carcinomas from patients with biallelic *MUTYH* mutations showed a strong supranuclear cytoplasmic staining without epithelial nuclear staining. The strong supranuclear staining was also observed in the three samples from patients with a single *MUTYH* mutation and in nine out of 11 samples from patients without *MUTYH* mutations, with or without nuclear staining. Samples incubated with the monoclonal antibodies showed a non-specific pattern. Our results demonstrate that, in contrast with previous data, the cytoplasmic staining in neoplastic cells does not discrimi-

nate *MUTYH* mutated from unmutated cases. At present, IHC cannot be used in clinical practice to differentiate between colorectal tissue with and without germline *MUTYH* mutations.

Keywords *MUTYH* · Polyposis · Colorectal carcinoma · Immunohistochemistry

Abbreviations

CRC	Colorectal cancer
<i>MUTYH</i>	MUTY homolog
MAP	<i>MUTYH</i> -associated polyposis
BER	Base excision repair
IHC	Immunohistochemistry
MSS	Microsatellite stable

Introduction

MUTYH-associated polyposis (MAP) is an autosomal-recessive disease characterized by multiple colorectal adenomas and cancer [1]. Approximately 30% of patients with more than 15 adenomas that do not carry pathogenic *APC* mutations are biallelic *MUTYH* mutation carriers [2]. MAP was first reported in a British family, in which three affected siblings were compound heterozygote for *MUTYH* mutations [1]. *MUTYH* acts together with *OGG1* and *MTH1* in the base excision repair (BER) system, a repair system to defend cellular DNA against the mutagenic effects of 7,8-dihydro-8-oxoguanine (8-oxoG) [2, 3]. 8-OxoG easily mispairs with adenine residues leading to G:C→T:A transversion mutations in the daughter strand [1, 4]. Normally, *MUTYH* is expressed in mitochondria and in the nuclei of human cells [5].

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Identification of patients with biallelic *MUTYH* mutation-associated polyposis is important to target effective preventive measures for patients and their families, which may lead to reduction in CRC-related mortality. DNA mutation analysis can determine the possible genetic cause of polyposis. To avoid expensive, unnecessary, and time-consuming DNA mutation analyses, there is a need for a screening test to select individuals eligible for DNA mutation analysis.

Immunohistochemical analysis is a rapid and inexpensive method, useful for a wide range of diseases. In a recent study by Di Gregorio et al., immunohistochemical staining of *MUTYH* protein was performed to identify patients with MAP [6]. A specific pattern of staining for the *MUTYH* protein was seen; unlike in patients without *MUTYH* mutations, patients with biallelic *MUTYH* mutations showed absence of nuclear staining and segregation of immune reactivity in the cytoplasm (supranuclear staining), both in neoplastic and surrounding healthy mucosa [6]. Therefore, tissues from patients with and without mutations might be distinguished from each other. Consequently, IHC could be used to identify patients with *MUTYH*-associated polyposis. The aim of this study is to further investigate the possibilities of immunohistochemistry as a pre-screening test to select patients for *MUTYH* mutation analysis.

Materials and methods

The study included 20 samples from 19 patients, divided into three groups. Samples were collected in five different pathology laboratories in different hospitals in the Netherlands (Radboud University Nijmegen Medical Center; Rijnsdijk Hospital, Arnhem; Amphia Medical Center, Breda; Jeroen Bosch Hospital, Den Bosch; Medisch Spectrum Twente, Enschede).

Group 1 consists of five patients carrying biallelic *MUTYH* mutations (six samples from colorectal carcinoma or adenoma); an overview of the clinical features and mutations of these patients is given in Table 1. All patients were compound heterozygous for pathogenic mutations in *MUTYH*. Mutation analysis of *MUTYH* was performed, in the Leiden University Medical Center, as described by Nielsen et al. [7], with sequence analysis of exon 1 till 16. Group 2 consists of 11 patients with polyposis or CRC without detectable mutations in *MUTYH* (11 samples from adenoma or CRC). Group 3 consists of three patients carrying a monoallelic *MUTYH* mutation (with three samples from adenomas and normal mucosa).

Immunohistochemical staining was performed on 4- μ m-thick, formalin-fixed, paraffin-embedded tissue sections that were prepared on coated slides and dried for 30 min

Table 1 Clinical features of patients with *MUTYH*-associated polyposis, the type biallelic mutations, and the immunoreactivity pattern of *MUTYH* protein, using the Abcam antibody

Patient	Number of adenomas	Sex	Age	Analyzed samples	Grading/differentiation ^a	Site	Mutation <i>MUTYH</i>	Type of mutation	Supranuclear staining ^b	Epithelial nuclear staining ^c
1	>100	M	48	Adenoma cancer (T4N0)	Low grade G2	Asc. colon sigmoid	c.697C>T, p. Arg233X c.1172C>T, p.Pro391Leu	Nonsense Missense	1	0
2	>50	F	47	Adenoma	Low grade	Rectum	c.494A>G, p. Tyr165Cys c.1172C>T, p.Pro391Leu	Missense Missense	1	0
3	50	M	48	Cancer (T3N0)	G2	Cecum	c.494A>G, p. Tyr165Cys c.1145G>A, p.Gly382Asp	Missense Missense	1	0
4	40–50	M	46	Cancer (T3N0)	G2	Cecum	c.697C>T p. Arg233X c.1145G>A p.Gly382Asp	Nonsense Missense	1	0
5	>10	M	48	Adenoma	Low grade	Desc. colon	c.1145G>A, p.Gly382Asp c.1172C>T, p.Pro391Leu	Missense Missense	1	0

^aDifferentiation for carcinomas: *G1* well differentiated, *G2* moderately differentiated, *G3* poorly differentiated, *G4* undifferentiated

^b0=absent, 1=present

^c0=no staining, 1=minimal to mild staining (<10–50% section *MUTYH* positive), 2=strong staining (>50% section *MUTYH* positive)

at 55°C. Tissue sections were deparaffinized in xylene and rehydrated with alcohol. Antigen retrieval was done by boiling in 10 mM citrate buffer (PH 6) for 10 min at 95°C. Endogenous peroxidase activity was blocked by exposing the slides to 3% H₂O₂ in methanol for 10 min. Then, sections were incubated with primary MUTYH antibody overnight at 4°C. Polyclonal MUTYH antibody (residues 531–546, Abcam, Cambridge, UK) at 1:300, primary polyclonal MUTYH antibody (residues 33–51, Calbiochem) at 1:1,600, and primary monoclonal MutYH antibody (clone 4D10, Abnova Corporation) at 1:200 were used. These dilutions were determined after examining several dilution series, to obtain the best results. Next, sections were incubated with Poly-HRP-GAM/R/R IgG for 30 min. Visualizing was done with DAB for 5 min. Nuclei were counterstained with hematoxylin. Slides were dehydrated, cleared in xylene, and mounted with micromount.

Normal immunoreactivity of the MUTYH protein was defined as the presence of nuclear and light cytoplasmic staining. Altered expression was considered when the cells showed disappearance of staining from the nucleus and instead showed supranuclear staining. Staining for MUTYH in the nucleus was evaluated by the scoring system Gao et al. reported: 0=no staining; 1=minimal to mild staining (10–50% section positive); 2=strong staining (>50% section positive) [8]. Cytoplasmic staining was classified as present or absent.

Results

With the polyclonal antibody, adenomas and carcinomas of all patients with *MUTYH* biallelic mutations showed strong supranuclear cytoplasmic staining, without nuclear expression of protein (Table 1). Adjacent normal mucosa, in patients with biallelic *MUTYH* mutations, showed the same pattern of expression found in adenomas and carcinomas. As shown in Fig. 1, supranuclear cytoplasmic staining was localized at the apex of the colonocytes (a) or neoplastic cells (b). The 11 samples of colorectal tissue of patients without *MUTYH* mutations, incubated with the polyclonal antibody, showed several patterns (Table 2 and Fig. 2). Nine samples showed the supranuclear cytoplasmic staining described above of which five had weak and four no epithelial nuclear staining. Two samples did not show the supranuclear cytoplasmic staining; they showed weak nuclear and weak cytoplasmic staining. All three samples of colon tissue of patients with one *MUTYH* mutation showed supranuclear staining; additionally, two showed also nuclear staining.

Diffuse cytoplasmic staining was observed in some samples either with or without *MUTYH* mutations; the intensity was always weak. From these results, we conclude that we could not differentiate between tissue with or

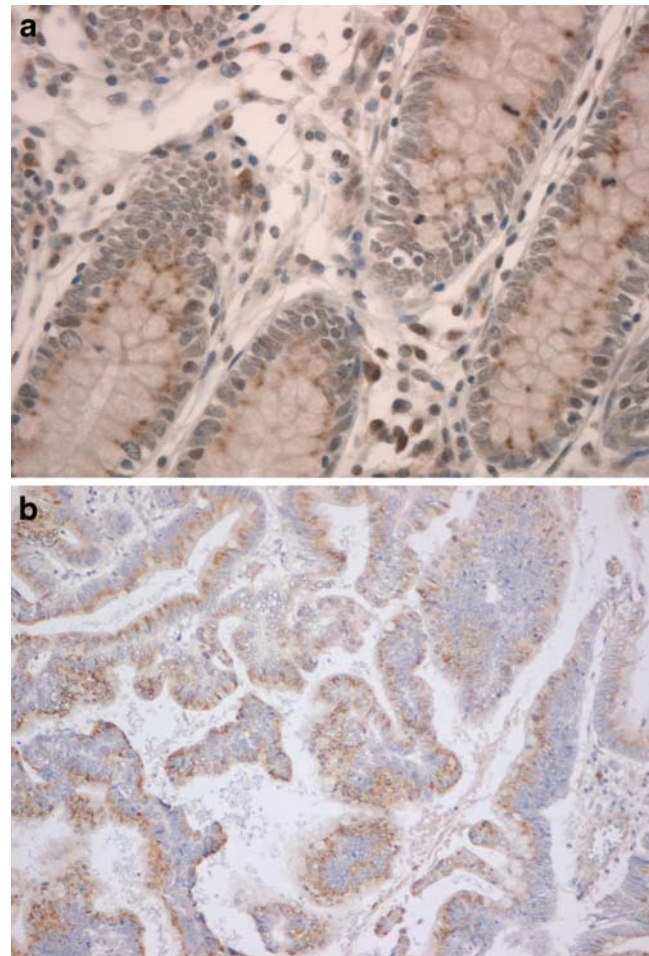


Fig. 1 Strong epithelial supranuclear cytoplasmic immunoreactivity and absence of nuclear expression of MUTYH protein in **a** normal mucosa (original magnification, $\times 500$) and **b** carcinoma (original magnification, $\times 100$) of patients with biallelic *MUTYH* mutations. Note nuclear staining in some stromal fibroblasts

without *MUTYH* mutations, while using the polyclonal antibody.

Two monoclonal antibodies were used to evaluate the MUTYH protein staining pattern as well. Samples incubated with the Calbiochem antibody showed strong nuclear and cytoplasmic staining for all tissue regardless whether *MUTYH* mutations were present. Samples incubated with the Abnova antibody showed no epithelial nuclear or cytoplasmic staining at all; however, nuclear staining was observed in stroma cells. This pattern was the same for tissue with and without *MUTYH* mutations.

Discussion

Using the commercially available polyclonal antibody for *MUTYH*, cytoplasmic staining in neoplastic cells does not discriminate *MUTYH*-mutated samples from unmutated

Table 2 Clinical features of patients without *MUTYH*-associated polyposis, the mutations tested, and the immunoreactivity pattern, using the Abcam antibody

Patient	Number of adenomas	Sex	Age	Analyzed samples	<i>MUTYH</i> mutations	<i>APC</i> mutations	Mis-match repair deficiency	PTEN	Supranuclear staining ^a	Epithelial nuclear staining ^b
Polyposis										
6	>50	M	79	Cancer	Neg	Neg ^c	ND ^d	ND	1	0
7	>20	M	58	Adenoma	Neg	Neg	ND	ND	0	1
8	>10	M	62	Adenoma	Neg	Neg	ND	ND	1	1
9	>10	M	45	Adenoma	Neg	Neg	MSS ^e	ND	1	0
10	>10	M	60	Adenoma	Neg	Neg	MSS	ND	0	1
11	>10	F	44	Adenoma	Neg	Neg	MSS	ND	1	0
12	>10	F	56	Adenoma	Neg	Neg	MSS	ND	1	1
Non-polyposis										
13	10	M	61	Adenoma	Neg	ND	ND	ND	1	1
14	<10	F	41	Adenoma	Neg	ND	ND	ND	1	1
15	2	F	49	Adenoma	Neg	Neg	MSS	Neg	1	0
16	0	M	43	Normal tissue	Neg	Neg	MSS	Neg	1	1

^a 0=absent, 1=present

^b 0=no staining, 1=minimal to mild staining (<10–50% section *MUTYH* positive), 2=strong staining (>50% section *MUTYH* positive)

^c *Neg* tested negative

^d *ND* not determined

^e *MSS* microsatellite stable

cases; presence of nuclear staining excludes the *MUTYH* mutation, but is of limited specificity. Samples incubated with the Calbiochem or Abnova antibody showed a non-specific pattern since no differentiation was possible between tissue with and without *MUTYH* mutations. Consequently, the two other antibodies did not seem to work on formalin-fixed, paraffin-embedded tissue after using different pretreatments and dilutions. There are no indications that, with the present mutation analysis of *MUTYH*, mutations are being missed (reported by C.M.J. Tops, Center for Human and Clinical Genetics, Leiden University Medical Center).

There are just a few immunohistochemical studies of the *MUTYH* protein described. Di Gregorio et al. described that tissue of patients with biallelic *MUTYH* mutations showed absence of nuclear staining and segregation of immunoreactivity (supranuclear) in the cytoplasm [6]. Their hypothesis for this pattern was that the protein produced by the mutated gene could lack the capacity to transfer into the nucleus and remain trapped in the cytoplasm [6]. Our results confirm this finding, but importantly we show that this pattern of staining does not distinguish between tissue of patients with and without *MUTYH* mutations. Recently, O'Shea et al. published results more consistent with our own, showing *MUTYH* immunohistochemistry not discriminating controls, biallelic, and heterozygote *MUTYH* mutation carriers [9].

Koketsu et al. showed that loss of expression of the BER proteins, *MUTYH*, *MTH1*, and *NTH1* occurs in sporadic colorectal cancer [10]. Nuclear *MUTYH* immunoreactivity

was detected in only 57% of cases (46/81) [10]. They described that the presence of nuclear *MUTYH* expression showed a significant correlation with the T-stage of the tumor ($p=0.04$) [10].

Further, it is not clear whether *MUTYH* protein is always expressed in the nucleus. Boldogh et al. showed that the majority of *MUTYH* protein was distributed in the cytoplasm, which is in agreement with a mitochondrial association of *MUTYH*, and that in only a small percentage (3–5%) of the cells *MUTYH*-specific fluorescence was also localized to the nuclei [11]. These findings are in contrast with the data of Tsai-Wu et al. which suggest that the *MUTYH* protein is mainly nuclear specific, based on their own polyclonal rabbit antibodies [12]. Recently, it was shown by Van Puijenbroek et al. that somatic *KRAS2* mutation testing of carcinomas can successfully be used as a pre-screening test for germline *MUTYH* mutation analysis [13].

In conclusion, our results demonstrate that, in contrast with the findings of Di Gregorio et al., while using the same methods and two additional antibodies, cytoplasmic expression of the *MUTYH* protein is not specific for germline *MUTYH* mutation. At present, immunohistochemistry cannot be used in clinical practice to differentiate between colorectal tissue with and without *MUTYH* mutations.

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Conflict of interest statement We declare that we have no conflict of interest.

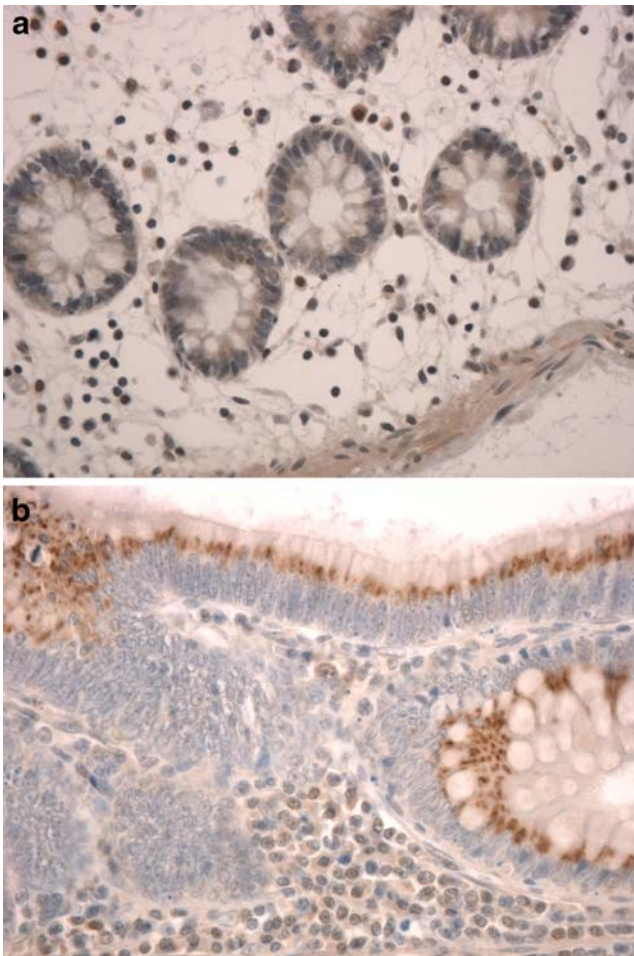


Fig. 2 Limited epithelial supranuclear cytoplasmic immunoreactivity and occasional nuclear expression of MUTYH protein in **a** normal mucosa but strong supranuclear and absent nuclear staining in **b** adenoma of patients without biallelic *MUTYH* mutations. Occasional nuclear staining in stromal cells (original magnification, $\times 500$)

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