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Elevated expression of p53 in early colon polyps in a pig model of human familial adenomatous polyposis

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Abstract

Familial adenomatous polyposis (FAP) is a hereditary predisposition to formation of colon polyps that can progress to colorectal cancer (CRC). The severity of polyposis varies substantially within families bearing the same germline mutation in the adenomatous polyposis coli (*APC*) tumour suppressor gene. The progressive step-wise accumulation of genetic events in tumour suppressor genes and oncogenes leads to oncogenic transformation, with driver alterations in the tumour protein p53 (*TP53*) gene playing a key role in advanced stage CRC. We analysed groups of pigs carrying a truncating mutation in *APC* ($APC^{1311/+}$; orthologous to human $APC^{1309/+}$) to study the influence of *TP53* polymorphisms and expression on the frequency of polyp formation and polyp progression in early-stage FAP. Five generations of $APC^{1311/+}$ pigs were examined by colonoscopy for polyposis severity and development. A total of 19 polymorphisms were found in 5′-flanking, coding, and 3′ untranslated regions of *TP53*. The distribution of *TP53* genotypes did not differ between $APC^{1311/+}$ pigs with low (LP) and high (HP) number of colon polyps. p53 mRNA expression was analysed in distally located normal mucosa samples of wild-type pigs, $APC^{1311/+}$ LP and HP pigs, and also in distally located polyp samples histologically classified as low-grade (LG-IEN) and high-grade intraepithelial dysplastic (HG-IEN) from $APC^{1311/+}$ pigs. p53 mRNA expression was found to be significantly elevated in HG-IEN compared to LG-IEN samples ($p = 0.012$), suggesting a role for p53 in the early precancerous stages of polyp development.

Keywords Colorectal cancer · Pig · Large animal model · Familial adenomatous polyposis · *TP53*

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (Arnold et al. 2017). While most cases are sporadic patients with the hereditary condition familial adenomatous polyposis (FAP) are strongly predisposed to develop CRC (Yurgelun et al. 2015; Mokarram et al. 2017). Most forms of FAP are caused by dominant mutations within the adenomatous polyposis coli (*APC*) gene, an important regulator of the canonical Wnt signalling pathway. Different *APC* mutations affect the severity of FAP and the most severe phenotype is caused by a nonsense substitution at *APC* codon 1309 (Plawski et al. 2013). Interestingly, the degree of polyposis caused by the same *APC* mutation can also vary considerably between individuals, even within the same family (Giardiello et al. 1994; Crabtree et al. 2002). The source (s) of this variability is still unknown, but studies to identify the genetic determinants are difficult to perform in humans (Talseth-Palmer et al. 2013).

We previously generated a line of genetically modified pigs (German Landrace x Pietrain crossbred) carrying an APC^{1311} mutation (orthologous to human APC^{1309}) to model human FAP (Flisikowska et al. 2012). Several generations of $APC^{1311/+}$ animals have been examined, revealing that

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porcine polyps spread throughout colon and rectum, closely resemble those in early stage human FAP by localisation, anatomical, morphological, histological and molecular criteria (Flisikowska et al. 2017; Stachowiak et al. 2017). It is also apparent that different pigs with the same mutation can exhibit very different numbers of polyps, classified as low (LP) or high (HP) polyp animals.

The tumour suppressor p53 plays a central role in most cancers (Rivlin et al. 2011), and *TP53* is one of the most frequently mutated genes in human CRC (Esplin and Snyder 2014; Li et al. 2015). The vast majority of driver mutations affect the p53 DNA binding domain (DBD) (Sameer 2013). *TP53* mutations can abolish cell control functions and confer gain-of-function properties (Solomon et al. 2018). Impairment of p53 function results in uncontrolled proliferation in the critical adenoma—adenocarcinoma transition stage (Eshghifar et al. 2017). Immunohistochemical analysis of polyp samples of human FAP have shown that p53 expression increases in advance stage polyps undergoing adenoma–carcinoma transformation (Wang et al. 2013).

Here we report the identification of *TP53* germline polymorphisms and comparative analysis of p53 mRNA expression in large intestine mucosa of $APC^{1311/+}$ LP and HP pigs, as well as in early low-grade (LG-IEN) and high grade (HG-IEN) intraepithelial dysplastic polyps of $APC^{1311/+}$ pigs.

Materials and methods

Ethical statement

All experimental procedures involving $APC^{1311/+}$ pigs were approved by the Government of Upper Bavaria (permit number 2 55.2-1-54-2532-6-13) and performed according to the German Animal Welfare Act and European Union Normative for Care and Use of Experimental Animals (EU Directive 2010/63/EU).

Material

Searching for germline polymorphism was carried out in coding sequence, 5′- and 3′UTR, as well as in 5′-flanking region (promoter). Sequencing of cDNA (5′ and 3′UTR and the coding sequence) was carried out on samples derived from LP ($N = 12$), HP ($N = 10$), wild type German Landrace x Pietrain crossbred (WT, $N = 5$), Polish synthetic line (900, $N = 12$), Polish Large White (PLW, $N = 8$), Polish Landrace (PL, $N = 6$), Hampshire (Hamp, $N = 8$) and Pietrain (Pi, $N = 10$). The 5′-flanking sequence was studied in the following number of samples: 15 (LP), 10 (HP), 24 (WT), 12 (990), 31 (PLW), 28 (PL), 9 (Hamp) and 28 (Pi).

Expression of p53 was analysed in: (A) colon normal mucosa collected post-mortem from four wild-type control pig breeds, representing PLW ($N = 7$), PL ($N = 5$), Pi ($N = 7$) and Hamp ($N = 7$); (B) biopsies collected from distal colon of $APC^{1311/+}$ LP ($N = 20$) and HP ($N = 20$) pigs; and (C) LG-IEN ($N = 20$) and HG-IEN ($N = 20$) polyps (Fig. 1a, b) from $APC^{1311/+}$ pigs, classified according to the AJCC TNM staging system (Edge and Compton 2010).

Methods

Genomic DNA was extracted from whole blood using the Blood Mini isolation kit (A&A Biotechnology). Total RNA was isolated with the TriPure (Roche) according to the manufacturer's protocol and 200 ng RNA reverse transcribed using SuperScript IV reverse transcriptase (Invitrogen). PCR amplification of *TP53* 5′UTR, the entire coding region and 3′ UTR were performed using cDNA derived from normal mucosa of large intestine samples of $APC^{1311/+}$ and wild type pigs. PCR primer pairs were designed using PRIMER3+ software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Polymorphism analysis was performed by Sanger sequencing of PCR amplicons purified with FastAP™ Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Scientific) and sequenced using BigDye Terminator v 3.1 (Life Technologies) on the 3130 Genetic Analyzer (Applied Biosystems) and analysed using LasergeneSeqMan Software (DNASTAR). The odds ratio value of allele and genotype frequencies was estimated with https://www.medcalc.org/calc/odds_ratio.php.

STARORF software (http://star.mit.edu/orf/) was used to search for potential changes in amino acid sequences, and HAPLOVIEW software (https://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) was used to perform haplotype analysis. Prediction of microRNA binding sites in 3′UTRs was based on the TARGET SCAN HUMAN 6.2 (http://targetscan.org/), miRBase (http://www.mirbase.org/cgi-bin/blast.pl), Segal Lab online microRNA prediction tool (https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) and miRIAD database (http://bmi.ana.med.uni-muenchen.de/miriad/). Comparative human and pig sequence analysis was performed with BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Emboss Water (http://www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html). Binding sites for transcription factors in the *TP53* promoter region were predicted using MatInspectorv.3.8 (Genomatix software suite).

Measurements of relative *p53* mRNA expression were carried out triplicate using primers that hybridised to exons 4 and 6 (NCBI, NM_213824.3), using KAPA SYBR® FAST qPCR Master Mix (Sigma Aldrich) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The specificity of qPCR products was verified by melting curve analysis. Relative expression levels were normalised to endogenous ribosomal

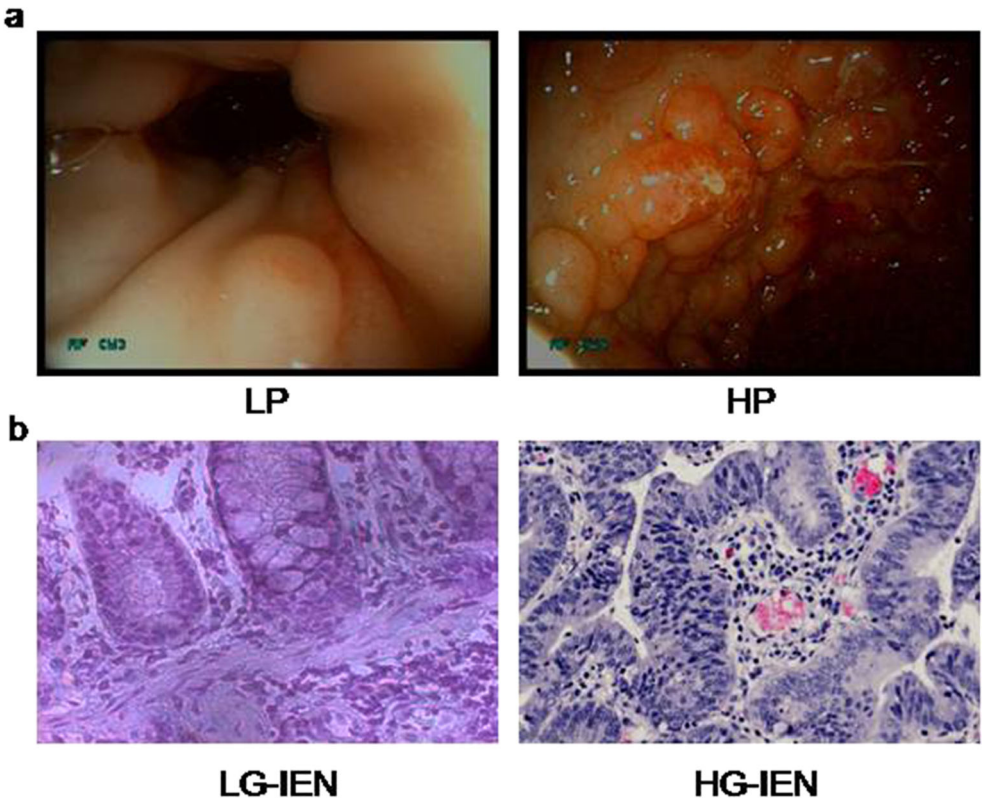

Fig. 1 **a** Endoscopic images of polyposis patterns specific for low- (LP) and high (HP) distal colon polyps of $APC^{I311/+}$ pigs. **b** Histopathology analysis of haematoxylin and eosin-stained (H&E) $APC^{I311/+}$ polyps sections of classified as low-grade (LG-IEN) and high-grade (HG-IEN) intraepithelial dysplastic adenomas according to the AJCC TNM staging system

protein S23 (*RPS23)* expression. A complete list of all primer sequences used is provided in Online Resource 1.

SigmaStatv.4.0 (SyStat software) was used for statistical analysis. Relative mRNA levels were compared between commercial breeds using non-parametric Kruskal-Wallis test. Statistical analyses of relative mRNA levels between LP and HP samples, and between LG-IEN and HG-IEN samples were performed using *t* test.

Results

Alignment of porcine *TP53* reference sequences (NC_010454.4) to human *TP53* (NC_000017.11) showed quite high similarity: 84% for coding sequence (1161 bp), 82% for the predicted amino acid sequence, 69% for promoter region (5′ flanking sequence, 1067 bp), 76% for 5′UTR (213 bp), and 45% for 3′UTR (548 bp).

A total of 19 SNPs were found: seven in the promoter region, ten in the coding sequence and two in the 3′UTR (Table 1). These included two novel polymorphisms, one in the coding region (ss3035653869) and one in the promoter (ss3035653873). In silico analysis indicated that three SNPs in the *TP53* promoter region are located within potential binding sites for the transcription factors Ets2 and TP53 (Table 1). Of the polymorphisms in the coding sequence, three were missense substitutions: rs333840391 (p.Glu2Asp), rs345539529 (p.Ser4Ala) and ss3035653869 (p.Pro271Leu). Two of these substitutions (rs333840391, rs345539529) were localised in p53 transactivation domain (TAD), whereas, the third SNP (ss3035653869) was in p53 DNA-binding domain (DBD). The distribution of minor missense variants at two sites (c.6T and c.10G), co-segregating as two haplotypes (G-T and T-G), occurred with a low frequency (≤ 0.02) in LP, HP, WT, PLW and Pi (Table 2). The third polymorphism (c.812C > T) was found in a single pig of line 990, only. Two breeds (PL and Hamp) were monomorphic at all 3 sites. Majority (6 of 7) of SNPs in 5′flanking region were found in PLW breed. In the 3′ UTR two SNPs were observed in Pietrain and 990 line, only.

Ten polymorphisms were detected in $APC^{I311/+}$ HP and LP pigs: one SNP in the promoter (rs343616038) and nine SNPs in coding sequence (rs333840391-p.Glu2Asp, rs345539529-p.Ser4Ala, rs81211694, rs345021946, rs324980623, rs334394956, rs81211695, rs318253531, rs81211696). The genotype distribution did not differ significantly between the HP and LP groups analysed.

Analysis of p53 mRNA expression in normal mucosa samples, collected from distal part of large intestine, showed no significant difference between wild-type (Fig. 2a) and $APC^{I311/+}$ LP and HP pigs (Fig. 2b). However, we observed 1.79-fold higher p53 expression ($p = 0.012$) in HG-IEN polyps compared to LG-IEN polyps (Fig. 2c). A similar, but not statistically significant, tendency was observed between samples of normal mucosa from $APC^{I311/+}$ LP and HP pigs (Fig. 2b).

Table 1 Polymorphisms identified in *TP53*

Region	Polymorphism	Predicted effect
5′ Flanking region	rs713924941	None
	rs696639821	ETS transcription factor
	rs334961490	None
	rs343616038	ETS transcription factor
	rs322319436	TP53 transcription factor [a]
	rs344321510	None
	ss3035653873 [b]	None
Coding exons		
Exon 2	rs333840391	p.Glu2Asp
Exon 2	rs345539529	p.Ser4Ala
Exon 4	rs81211694	Silent substitution
Exon 6	rs345021946	Silent substitution
Exon 7	rs324980623	Silent substitution
Exon 7	rs334394956	Silent substitution
Exon 8	ss3035653869 [b]	p.Pro271Leu
Exon 8	rs81211695	Silent substitution
Exon 8	rs318253531	Silent substitution
Exon 10	rs81211696	Silent substitution
3′ UTR		
Exon 11	rs336819886	Alteration of target sequence for miR-6
Exon 11	rs332809145	Alteration of target sequence for miR-524-3

[a] (Wang and El-Deiry 2006)

[b] Novel

Discussion

The identification of genetic factors that influence the number of polyps that develop and the tendency for polyps to progress towards cancer would aid in monitoring individuals at risk of developing CRC and could inform the development of novel treatments. We compared groups of pigs that carried the same initiating *APC* mutation but exhibited different levels of polyposis to investigate whether polymorphism and expression of *TP53* gene was a determining factor.

In silico analysis revealed that the regulatory region of *TP53* showed greater homology between human and pig than between human and mouse. Two SNPs (rs696639821, rs343616038) localised in the 5′-flanking region possibly change the binding affinity for the Ets (Erythroblastosis proto-oncogene) transcription factor. Interestingly, Ets is highly expressed in colon epithelium (Jedlicka and Gutierrez-Hartmann 2008). Three missense SNPs were found in the porcine *TP53* coding region, two of which (rs333840391, p.Glu2Asp and rs345539529, p.Ser4Ala) lie within the N-

Table 2 Frequencies of missense variants of *TP53* in the studied cohorts of pigs

Lines and breeds	*N*	rs333840391 (c.6G > T, p.Glu2Asp)		rs345539529, (c.10T > G, p.Ser4Ala)		ss3035653869, (c.812C > T, p.Pro271Leu)	
		G	T	T	G	C	T
WT	5	0.8	0.2	0.8	0.2	1	0
LP	12	0.87	0.13	0.87	0.13	1	0
HP	10	0.8	0.2	0.8	0.2	1	0
990	12	1	0	1	0	0.96	0.04
PLW	8	0.94	0.06	0.94	0.06	1	0
PL	6	1	0	1	0	1	0
Hamp	8	1	0	1	0	1	0
Pi	10	0.75	0.25	0.8	0.2	1	0

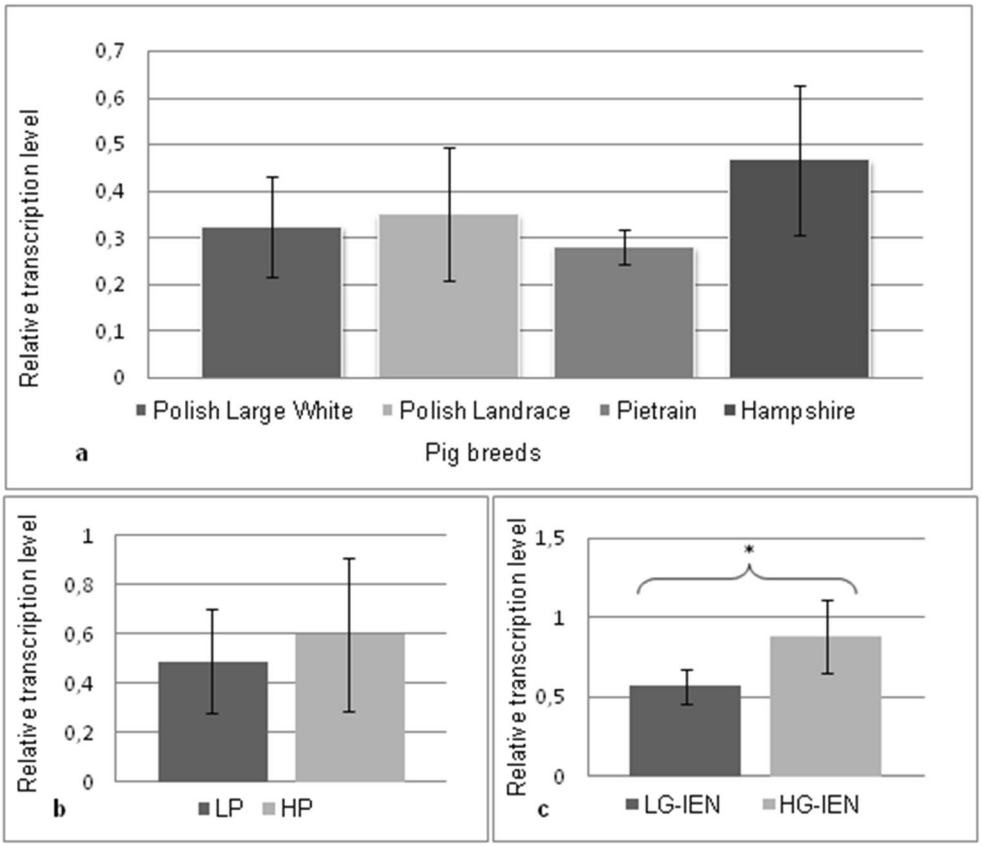

Fig. 2 Comparison of relative p53 mRNA levels in **a** normal mucosa of wild-type pig breeds; **b** normal mucosa of $APC^{1311/+}$ low polyposis (LP) and high polyposis (HP) pigs; **c** low-grade (LG-IEN) and high-grade (HG-IEN) intraepithelial dysplastic polyps of $APC^{1311/+}$ pigs. * - values differ significantly ($p = 0.012$)

terminal transactivation domain (TAD). This domain covers the first 61 amino acids harbouring two activation subdomains (AD) (AD1: 18-26 aa and AD2: 44-54 aa), recognised by mouse double minute protein 2 (*MDM2*), an important negative regulator of *TP53* (Krois et al. 2016). The impact of these polymorphisms on TAD function merits further study.

The third missense polymorphism (ss3035653869) was localised in exon 8 and causes p.Pro271Leu substitution in the DBD, which is encoded by porcine exons 5-8. Approximately, 80% of mutations in the human coding sequence have been transitions within "hotspot" codons in the DBD (El-Mahdani et al. 1997). Interestingly, the p.Pro271Leu is orthologous to human p.Pro278Leu polymorphism (rs876659802) reported as pathogenic variant of *TP53* for various types of cancer (ClinVar ID232497, https://www.ncbi.nlm.nih.gov/clinvar?term=rs876659802). Moreover, Abaigar et al. (2016) indicate an association of the p.Pro278Leu with complex chromosomal alterations (chromothripsis), a phenomenon observed in many tumours, including colorectal cancer. The DNA mutations at codon 278 are also associated with breast cancer outcome in humans (Chitrala and Yeguvapalli 2014).

Epidemiologic studies carried out in humans showed that different molecular pathways are responsible for development of CRC, depending on proximal (right sided) or distal (left sided) polyps localization (Lee et al. 2015). Our comparison of polyps, collected from distal part of colon, at different stages of development revealed that *TP53* mRNA expression was higher in HG-IEN than LG-IEN polyps. Correlation between polyposis severity and the proportion of high-grade dysplasia has been reported in human FAP patients (Shussman and Wexner 2014). Moreover, it is known, that distally located polyps are at higher risk of transformation into colorectal cancer, malignancies and chromosomal instability, while compared with proximal polyps (Minoo et al. 2010; Syngal et al. 2015). In humans, an increased expression of p53 mRNA and protein has been observed in the advanced stages of CRC and during colonic adenoma-adenocarcinoma transformation (El-Mahdani et al. 1997; Wang et al. 2013). Furthermore, López et al. (2012) indicated that the p53 expression was increased in CRC samples and highly correlated with missense mutations, although, localization of the tumours was irrelevant. While increased p53 expression in advanced polyps is associated with a strong risk of malignant transformation (Kaklamanis et al. 1993), such correlations have not so far been reported in early stage polyps. In mice with partially deleted *Apc*, p53 expression is only moderately elevated in colon polyps (Hinoi et al. 2007). Finally, it was suggested that disclosure of the elevated p53 mRNA level in distal colon-derived polyps indicates its prognostic value (Da-Zhong Cao et al. 2017).

Mutations in the *TP53* gene are extremely common across a wide range of tumour types (Kandoth et al. 2013); hence the interest in treatments to restore normal p53 function for a wide variety range of human cancers (Bykov et al. 2018). Our study indicates a function for *TP53* function even in early stages of CRC development.

Modelling polyposis and CRC in pigs offers a useful additional resource to mouse models to study the pathobiology of polyposis and CRC (Perleberg et al. 2018). The $APC^{1311/+}$ pig allows human scale procedures such as endoscopic monitoring and polyp biopsy, and enables comparative analyses that are not possible in human populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experimental procedures involving $APC^{1311/+}$ pigs were approved by the Government of Upper Bavaria (permit number 2 55.2-1-54-2532-6-13) and performed according to the German Animal Welfare Act and European Union Normative for Care and Use of Experimental Animals (EU Directive 2010/63/EU). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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