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Up and downregulation of p16^{Ink4a} expression in *BRAF*-mutated polyps/adenomas indicates a senescence barrier in the serrated route to colon cancer

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P16^{Ink4a} is an important factor in carcinogenesis and its expression can be linked to oncogene-induced senescence. Oncogene-induced senescence is characterized by growth arrest and occurs as a consequence of oncogene activation due to *KRAS* or *BRAF* mutation. It has been shown that the induction of p16^{Ink4a} in premalignant lesions and its loss during malignant transformation is an important mechanism in the carcinogenesis of several tumours. Loss of p16^{Ink4a} is often caused by *CDKN2A* promoter hypermethylation. This mechanism of gene silencing is associated with the CpG island methylator phenotype (CIMP) in colorectal carcinomas, which is characterized by widespread promoter methylation. In particular, colorectal carcinomas with *BRAF* mutations have been shown to be strongly associated with CIMP. Also, *BRAF* mutations are strongly correlated with the serrated route to colorectal cancer. In this study, we investigated p16^{Ink4a} expression and promoter methylation in *BRAF*-mutated serrated lesions of the colon. P16^{Ink4a} expression was found to be upregulated in premalignant lesions and was lost in invasive serrated carcinomas. P16^{Ink4a} expression and Ki67 expression were mutually exclusive, indicating that p16^{Ink4a} acts as cell cycle inhibitor. Additionally, progression of malignant transformation in serrated lesions was accompanied by increasing methylation of the *CDKN2A* promoter. Therefore, our data provide evidence for oncogene-induced senescence in the serrated route to colorectal cancer with *BRAF* mutation and upregulation of p16^{Ink4a} expression appears to be a useful indicator of induction of senescence. Loss of p16^{Ink4a} expression occurs during malignant transformation and is caused mainly by aberrant methylation of the *CDKN2A* promoter.
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P16^{Ink4a} is a cell cycle inhibitor and an important factor in carcinogenesis. It controls p16^{Ink4a}-dependent growth arrest and its expression can be linked to oncogene-induced senescence.[1–3] Oncogene-induced senescence is characterized by an irreversible growth arrest and occurs as a consequence of oncogene activation due to *KRAS* or *BRAF* mutation.[4–6] The rupture of oncogene-induced senescence is an important mechanism for malignant transformation and can be caused by genetic alterations leading to a loss of function of p16^{Ink4a}. The induction of p16^{Ink4a} in premalignant lesions and its loss during malignant transformation have been demonstrated in the carcinogenesis of the liver, lung and pancreas.[7–9] Loss of p16^{Ink4a} is often caused by *CDKN2A* (p16^{Ink4a}) promoter hypermethylation.[10] This mechanism of gene silencing is associated with the CpG island methylator phenotype (CIMP) of colorectal cancer, which implies widespread promoter methylation including *CDKN2A*.[11–14] Especially, colorectal carcinomas with *BRAF* mutations

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have been shown to be strongly associated with CIMP.[15] In addition, *BRAF* mutations are strongly correlated with the serrated route to colorectal cancer comprising hyperplastic polyps, sessile serrated adenomas and traditional serrated adenomas as precancerous lesions.[16–18]

Most molecular studies of malignant transformation in the serrated route are dealing with promoter methylation of *hMLH1* and the development of mismatch repair deficiency as well as high-degree microsatellite instability after loss of MLH1 expression. However, there are few analyses focussing on $p16^{Ink4a}$. Up to now it is known that the expression of $p16^{Ink4a}$ is upregulated in hyperplastic polyps and sessile serrated adenomas[19] and that promoter methylation of *CDKN2A* can be observed in serrated carcinomas.[20,21] Our work is the first comprehensive analysis of the expression and promoter methylation of *CDKN2A* in the spectrum of serrated lesions of the colon with *BRAF* mutation. Our findings of an initial $p16^{Ink4a}$ upregulation in premalignant serrated lesions and of a methylation-associated $p16^{Ink4a}$ downregulation during malignant transformation indicate an induction and loss of an oncogene-induced senescence barrier in colon carcinogenesis initiated by *BRAF* mutation.

Materials and methods

Specimens

Formalin-fixed paraffin-embedded tissue from 98 serrated tumour cases was taken from the archives of the Department of Pathology, Ludwig-Maximillians Universität, München. All samples were classified by two independent observers (TK and LK) applying the criteria of Torlakovic *et al*.[22] Discrepancies were discussed until consensus was found. Our study enrolled hyperplastic polyps, sessile serrated adenomas without intraepithelial neoplasia, traditional serrated adenomas with low-grade intraepithelial neoplasia, sessile serrated adenomas with low-grade intraepithelial neoplasia, traditional serrated adenomas and sessile serrated adenomas with high-grade intraepithelial neoplasia and early invasive serrated ex-adenoma adenocarcinoma (pT1-category).

All cases were investigated with regard to *BRAF* and *KRAS* mutational status. Neither *KRAS* nor *BRAF* mutation was found in 15 cases. *KRAS* mutation was detected in 13 cases and *BRAF* mutation was found in 70 cases. *KRAS*-mutated lesions were enrolled in a previous study which was recently published.[23] For the final case collection only the 70 tumours with *BRAF* mutation were included.

Immunohistochemistry

Immunohistochemical staining was done on 5 μm tissue sections of formalin-fixed paraffin-embedded tumour samples. As primary antibodies, anti-$p16^{Ink4a}$ mouse monoclonal antibody (Diagnostic Biosystems, dilution 1:100) and anti-Ki67 monoclonal mouse antibody (DAKO, dilution 1:50) were used. Staining was performed on a Ventana Benchmark XT autostainer with the XT ultraView DAB Kit (Ventana Medical Systems). All slides were counterstained with Haematoxylin (Vector). To exclude unspecific staining, system controls were included. As positive controls for the immunohistochemistry, cervical intraepithelial neoplasia ($p16^{Ink4a}$) and palatine tonsil (Ki67) were used.

KRAS−/*BRAF* Mutational and *CDKN2A* Hypermethylation Analyses

Analyses of mutation of *KRAS* codon 12/13 and *BRAF*(p.V600E) were done as described before.[24] Briefly, genomic DNA was extracted from microdissected serrated lesions employing QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden). In early invasive serrated ex-adenoma adenocarcinoma only invasive areas were microdissected and in serrated lesions with IEN only the region of IEN was investigated. For *CDKN2A* hypermethylation analyses, DNA was deaminated by bisulphite treatment using EZ DNA Methylation-Gold Kit (HiSS Diagnostics GmbH, Freiburg). Pyro-sequencing was done by subjecting the purified DNA to a $p16^{Ink4a}$ methyl specific PCR employing Empyromark Q24 p16 kit (Qiagen) and the subsequent analysis of the PCR products on a Q24 pyrosequencing device (Qiagen) together with Pyro-Gold kit (Qiagen). The data were analysed using the PyroMark™ Q24 software according to the manufacturer's recommendations.[25,26] All reactions and procedures were carried out as recommended by the respective user's manual.

Hypermethylation Score

DNA of the colon cancer cell line HCT116 that is hemimethylated for the *CDKN2A* promoter was used as internal control. Grade of hypermethylation was scored as hypermethylated, partially hypermethylated or not unmethylated in comparison with methylation status of HCT116 DNA which was between 40 and 45% within all seven CpG islands. For each case, the arithmetic average was taken regarding methylation status of the seven CpG islands. Unmethylated was defined as 0–20% methylation, partial methylation was defined as 21–55% methlyation and hypermethylated was defined as 56–100% methylation.

Results

Classification and Clinical Data of *BRAF*-Mutated Serrated Lesions

The final case collection included 70 tumours with *BRAF* mutation. It comprised 20 hyperplastic polyp

Table 1 Distribution and average age of serrated lesions

Histology	*Number of cases* (n)	*Right sided* (%)	*Left sided* (%)	*Location n.a.* (%)	*Average age (years)*	*Age range (years)*
HP	20	8/20 (40)	12/20 (60)	0/20 (0)	64	34–89
SSA	24	15/24 (63)	9/24 (37)	0/24 (0)	65	47–85
SSA with LGIEN	2	1/2 (50)	1/2 (50)	0/2 (0)	64	57–69
SSA with HGIEN	8	5/8 (63)	0/8 (0)	3/8 (37)	69	62–77
TSA with LGIEN	10	4/10 (40)	5/10 (50)	1/10 (10)	75	63–89
TSA with HGIEN	1	0/1 (0)	1/1 (100)	0/1 (0)	80	80
Invasive Carcinoma	5	0 (0)	0 (0)	5/5 (100)	70	66–73

HP, hyperplastic polyp; SSA, sessile serrated adenoma; TSA, traditional serrated adenoma; LGIEN, low-grade intraepithelial neoplasia; HGIEN, high-grade intraepithelial neoplasia; n.a., not applicable.

(17 microvesicular type, 2 mucin-poor and 1 goblet-cell-rich), 24 sessile serrated adenomas without IEN, 2 sessile serrated adenomas with low-grade intraepithelial neoplasia, 8 sessile serrated adenomas with high-grade intraepithelial neoplasia, 10 traditional serrated adenomas with low-grade intraepithelial neoplasia, 1 traditional serrated adenoma with high-grade intraepithelial neoplasia and 5 early invasive serrated ex-adenoma adenocarcinoma. Details about the location and age of patients are given in Table 1.

p16Ink4a Expression and Hypermethylation of the *CDKN2A* Promoter

Immunohistochemical p16Ink4a expression is absent in normal colonic mucosa (Figure 1a). An upregulation of p16Ink4a can be detected in premalignant serrated lesions with *BRAF* mutation. A low and high expression pattern can be distinguished. The low expression pattern is characterized by an immunostaining of p16Ink4a in epithelial cells at the base of the crypts or in randomly distributed tiny spots of epithelial cells of the *BRAF*-mutated serrated lesions (Figure 1b). The high expression pattern is defined by confluent areas of epithelial cells with very strong immunostaining of p16Ink4a (Figure 1c).

The low expression pattern occurs in all hyperplastic polyps (100%), in the majority of sessile serrated adenomas (71%) and in almost half of the traditional serrated adenomas (45%) (Table 2). The high expression is seen only in sessile serrated adenomas and traditional serrated adenomas, but not in hyperplastic polyps. It is found more frequently in traditional serrated adenomas (37%) than in sessile serrated adenomas (15%). Few sessile serrated adenomas (15%) and traditional serrated adenomas (18%) and all early invasive adenocarcinoma (100%) are p16Ink4a negative.

Up and downregulation of p16Ink4a expression correlates with the presence of IEN and invasiveness. Almost all serrated lesions without IEN (93%) show low p16Ink4a expression (Table 3). High expression occurs mainly in adenomas with low-grade intraepithelial neoplasia (33%) or high-grade intraepithelial neoplasia (33%). On the other hand, loss of p16Ink4a is detected in more than half of polyps with high-grade intraepithelial neoplasia (56%) and in all early invasive ex-adenoma adenocarcinoma (100%). This indicates a switch from up to downregulation of p16Ink4a in the phase of high-grade intraepithelial neoplasia.

Immunohistochemical p16Ink4a expression correlates with hypermethylation of the *CDKN2A* promoter. No methylation is seen in normal mucosa and in the majority of hyperplastic polyps (80%) (Table 2). There is an increase of partial methylation or hypermethylation in sessile serrated adenomas (53%) compared with hyperplastic polyps (20%). Hypermethylation is most frequently observed in traditional serrated adenomas (45%) and early invasive adenocarcinoma (100%). Moreover, the detection of hypermethylation is very low in lesions without IEN (5%), increases in lesions with low-grade intraepithelial neoplasia (33%), occurs frequently in lesions with high-grade intraepithelial neoplasia (67%) and characterizes all lesions of early invasive adenocarcinoma (100%) (Table 3).

During serrated tumorigenesis from normal colonic mucosa to early invasive adenocarcinoma p16Ink4a expression and methylation status starts with p16Ink4a negativity without *CDKN2A* methylation, which is the finding of normal mucosa (100%) (Table 4). The end point of the serrated route is p16Ink4a negativity with *CDKN2A* promoter hypermethylation, which occurs in some sessile serrated adenomas (15%) and traditional serrated adenomas (18%) and in all early invasive adenocarcinoma (100%). In between, hyperplastic polyps, sessile serrated adenomas and traditional serrated adenomas exhibit low or high p16Ink4a expression with an increasing percentage of high expression from hyperplastic polyps (0%) to sessile serrated adenomas (15%) and to traditional serrated adenomas (37%) (Table 4).

Correlation of p16Ink4A Expression and the Ki67 Proliferation Index

Immunohistochemical Ki67 staining as an index of proliferation and p16Ink4a expression are mutually exclusive in serrated lesions (Figure 2a–h).

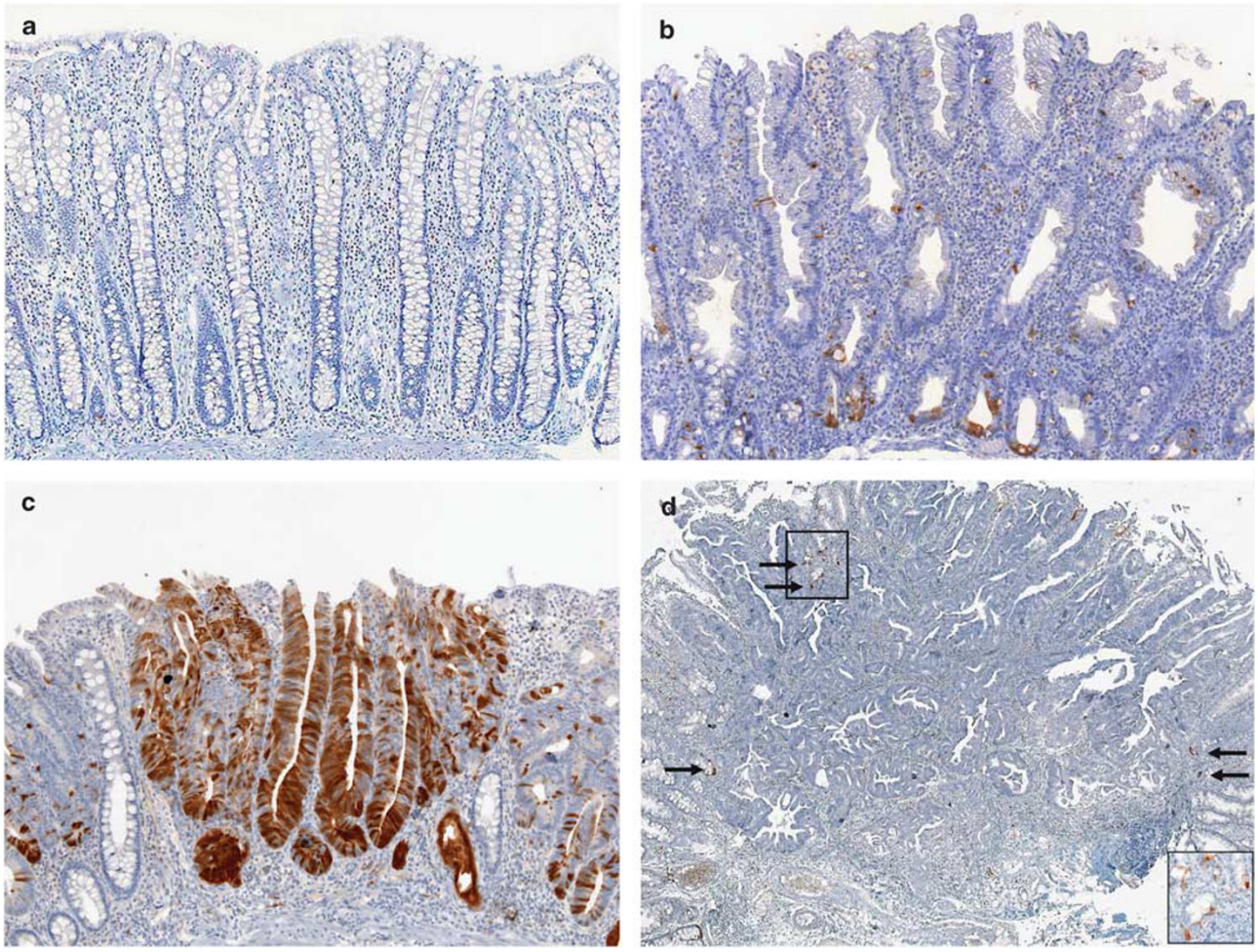

Figure 1 p16^{Ink4a} expression in the serrated pathway. P16^{Ink4a} expression is negative in normal colorectal mucosa (**a**). In hyperplastic polyps and sessile serrated adenomas, a low expression pattern of p16^{Ink4a} is found. The low expression pattern is characterized by cytoplasmic and nuclear immunostaining of p16^{Ink4a} in epithelial cells at the base of the crypts or in randomly distributed tiny spots of epithelial cells (**b**). In sessile and traditional serrated adenomas with low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia a high expression pattern of p16^{Ink4a} is seen. The high expression pattern shows confluent areas of epithelial cells with very strong cytoplasmic and nuclear immunostaining of p16^{Ink4a} (**c**). In invasive carcinomas p16^{Ink4a} expression is lost (**d**) and only adjacent adenomatous crypts display p16^{Ink4a}-positive cells (arrows, inset).

Table 2 p16^{Ink4a} expression and methylation status in normal mucosa and serrated lesions of the colon

Histology	*Number of cases* (n)	*p16 Negative* (%)	*p16 Low* (%)	*p16 High* (%)	*Unmethylated* (%)	*Partial methylation* (%)	*Hypermethylation* (%)
Normal mucosa	15	15/15 (100)	0/15 (0)	0/15 (0)	15/15 (100)	0/15 (0)	0/15 (0)
HP	20	0/20 (0)	20/20 (100)	0/20 (0)	16/20 (80)	3/20 (15)	1/20 (5)
SSA	34	5/34 (15)	24/34 (71)	5/34 (15)	16/34 (47)	12/34 (35)	6/34 (18)
TSA	11	2/11 (18)	5/11 (45)	4/11 (37)	6/11 (55)	0/11 (0)	5/11 (45)
Invasive carcinoma	5	5/5 (100)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	5/5 (100)

Table 3 p16^{Ink4a} expression and methylation status in serrated lesions of the colon in correlation to IEN

Histology	*Number of cases* (n)	*p16 Negative* (%)	*p16 Low* (%)	*p16 High* (%)	*Unmethylated* (%)	*Partial methylation* (%)	*Hypermethylation* (%)
Serrated lesions without IEN	44	1/44 (2)	41/44 (93)	2/44 (5)	29/44 (65)	13/44 (30)	2/44 (5)
Serrated lesions with LGIEN	12	1/12 (8)	7/12 (58)	4/12 (33)	8/12 (67)	0/12 (0)	4/12 (33)
Serrated lesions with HGIEN	9	5/9 (56)	1/9 (11)	3/9 (33)	1/9 (11)	2/9 (22)	6/9 (67)
Invasive carcinoma	5	5/5 (100)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	5/5 (100)

IEN, intraepithelial neoplasia; LGIEN, low-grade intraepithelial neoplasia; HGIEN, high-grade intraepithelial neoplasia.

Table 4 Correlation of histology with up and downregulation of $p16^{INK4a}$ expression

Histology	*Number of cases* (n)	*p16 Negative unmethylated (%)*	*p16 Low*[a] *(%)*	*p16 High*[a] *(%)*	*p16 Negative hypermethylated (%)*
Normal mucosa	15	15/15 (100)	0/15 (0)	0/15 (0)	0/15 (0)
HP	20	0/20 (0)	20/20 (100)	0/20 (0)	0/20 (0)
SSA	34	0/34 (0)	24/34 (70)	5/34 (15)	5/34 (15)
TSA	11	0/11 (0)	5/11 (45)	4/11 (37)	2/11 (18)
Invasive carcinoma	5	0/5 (0)	0/5 (0)	0/5 (0)	5/5 (100)

HP, hyperplastic polyp; SSA, sessile serrated adenoma; TSA, traditional serrated adenoma.
[a]Includes unmethylated, partial methylated and hypermethylated serrated lesions.

Accordingly, in polyps with low $p16^{Ink4a}$ expression, which is mainly confined to the base of the crypts, the zone of proliferation moves towards the middle compartment of the elongated serrated crypts with no Ki67 staining at the base of the crypts (Figure 2c and d). An almost complete loss of Ki67 staining is seen in lesions with a high expression of $p16^{Ink4a}$ defined by confluent areas of positive cells (Figure 2e and f). Conversely, early invasive adenocarcinomas with loss of $p16^{Ink4a}$ expression are characterized by strong Ki67 staining indicating a high proliferation index.

Discussion

In this study, we demonstrate the absence of $p16^{Ink4a}$ expression in normal mucosa and its upregulation in *BRAF*-mutated serrated lesions of the colon. All hyperplastic polyps and the majority (84%) of sessile serrated adenomas and traditional serrated adenomas exhibit $p16^{Ink4a}$ expression. The expression of $p16^{Ink4a}$ and Ki67 is mutually exclusive in serial sections, indicating that $p16^{Ink4a}$ acts as cell cycle inhibitor. While all serrated lesions with low-grade intraepithelial neoplasia show low or high expression of $p16^{Ink4a}$, serrated lesions with high-grade intraepithelial neoplasia are characterized either by a high expression or loss of $p16^{Ink4a}$ expression. All ex-adenoma serrated adenocarcinoma show a loss of $p16^{Ink4a}$ expression. This indicates a final loss of $p16^{Ink4a}$ during the process of malignant transformation and an up and downregulation of $p16^{Ink4a}$ in the serrated route to colon cancer.

Our observations are similar to investigations concerning human naevi where the concept of oncogene-induced senescence associated with *BRAF* mutation has been established. In melanocytic naevi, a stop of cell proliferation is correlated with a high $p16^{Ink4a}$ expression and found in combination with a frequent mutation in the *BRAF* oncogen. In the model proposed by Michaloglou *et al*,[6] *BRAF* signalling primarily causes benign naevi exhibiting a limited growth due to an induction of senescence. This senescence barrier must be overcome by further genetic alterations for the development of malignant melanoma. Our data indicate an analogous process in human serrated polyps where oncogene-induced senescence driven by initiating *BRAF* mutations might cause a growth arrest and stop further malignant transformation. The loss of $p16^{Ink4a}$ expression due to hypermethylation of the *CDKN2A* promoter might overcome the senescence barrier and could enable further progression into invasive colon cancer (Figure 3).

Interestingly, ultrastructural studies have already demonstrated features of senescence in serrated polyps.[21,27] Our recent study of a mouse model shows additional support for the concept of oncogene-induced senescence in the serrated route. In this model, intestinal cell-specific expression of oncogenic $K\text{-}ras^{G12D}$ induced murine serrated polyps, which were characterized by $p16^{Ink4a}$ overexpression and induction of senescence. Deletion of the Ink4a/Arf locus in these $K\text{-}ras^{G12D}$ mice prevented senescence and correlated with the development of invasive, metastasizing carcinomas. These tumours exhibited morphological and molecular alterations comparable to human *KRAS*-mutated serrated carcinomas. In human *KRAS*-mutated serrated carcinomas decreased expression or loss of $p16^{Ink4a}$ was associated with *CDKN2A* promoter methylation in correspondence with our findings in human *BRAF*-mutated serrated lesions.[23]

Furthermore, it was recently published that oncogenic $BRAF^{V600E}$ mice show gastrointestinal serrated carcinogenesis. After a phase of high proliferation, the lesions developed a state of oncogene-induced senescence. This was overcome by methylation of the *CDKN2A* promoter leading to the development of invasive carcinomas.[28] The mouse model confirms our findings in human serrated polyps.

Progression of malignant transformation in serrated lesions can be correlated with different degrees of *CDKN2A* promoter methylation. Serrated lesions without IEN or low-grade IEN exhibit no or only partial *CDKN2A* promoter methylation, whereas lesions with high-grade IEN or invasive carcinomas display in the majority of cases hypermethylation of the *CDKN2A* promoter. Therefore, the morphological stage of high-grade IEN can be correlated with a gradual loss of $p16^{Ink4a}$ expression

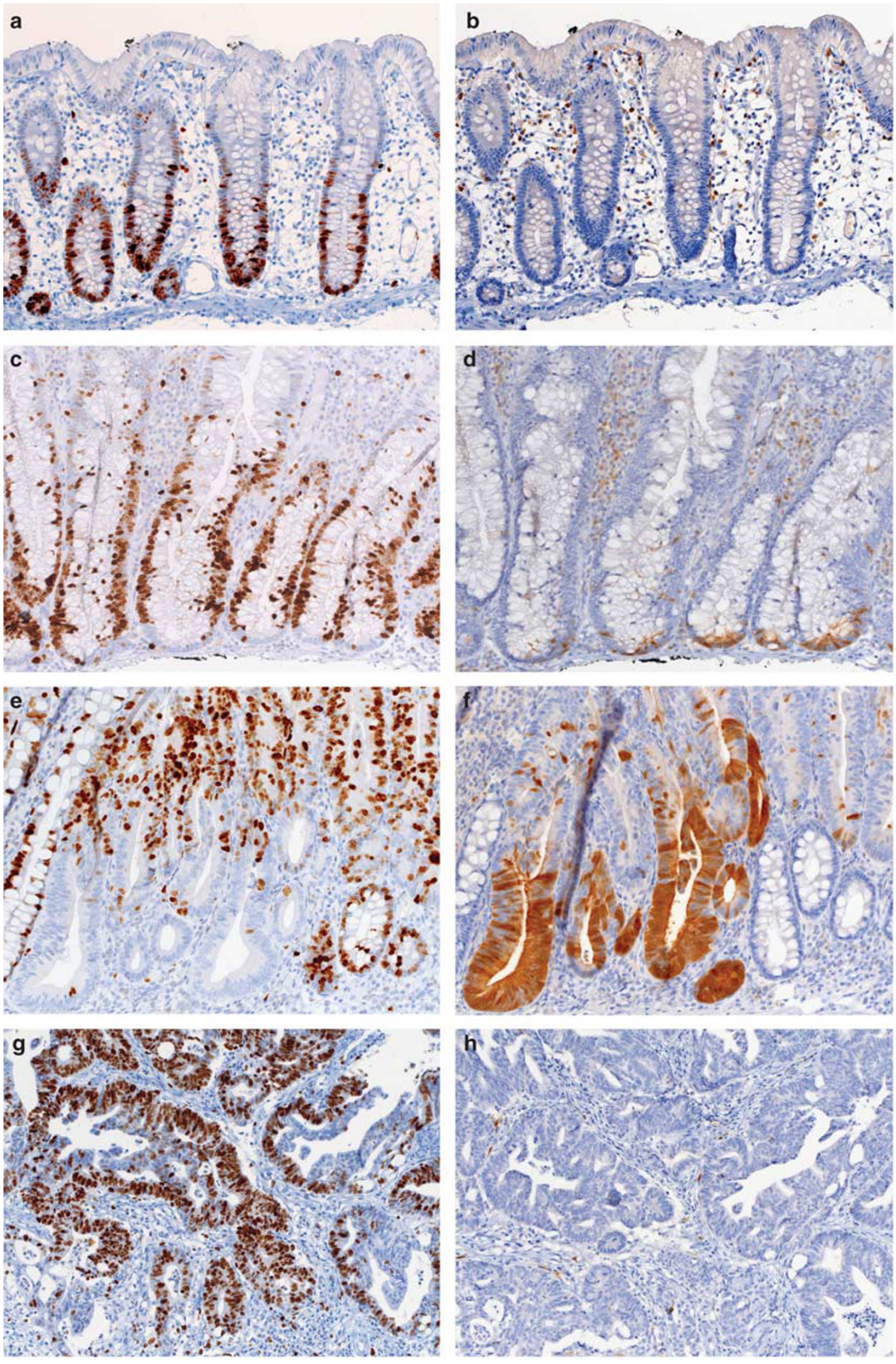

Figure 2 Correlation of p16^{Ink4a} and Ki67 expression. Normal mucosa (**a**, **b**) with Ki67-positivity in the basal third of the crypts (**a**) and lack of p16^{Ink4a} (**b**). Hyperplastic polyps (**c**, **d**) with loss of Ki67-positivity (**c**) in the basal part of elongated crypts corresponding to focal p16^{Ink4a} expression (**d**) (low-grade pattern) in this area. Sessile serrated adenomas with high-grade intraepithelial neoplasia (**e**, **f**) exhibiting a loss of Ki67 staining (**e**) in a confluent epithelial area with strong p16^{Ink4a} staining (high expression pattern) (**f**). Early invasive serrated adenocarcinoma (**g**, **h**) characterized by strong Ki67 staining (**g**) corresponding with the loss of p16^{Ink4a} expression (**h**).

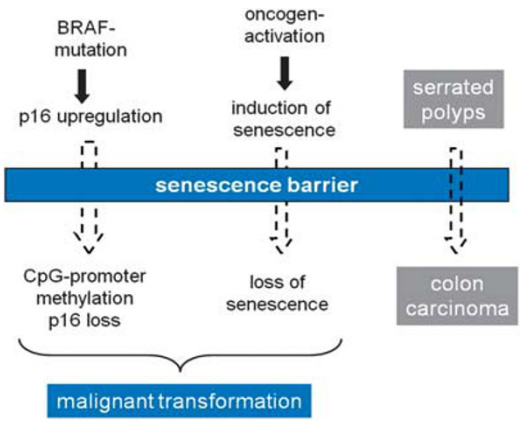

Figure 3 Model of a senescence barrier in the serrated route to colon cancer. In human serrated lesions/polyps, oncogene-induced senescence is induced by initiating *BRAF* mutations leading to premalignant lesions with an upregulation of $p16^{Ink4a}$ and a limitation of growth by a senescence barrier. Epigenetic silencing through aberrant methylation of the $p16^{Ink4a}$ promoter overcomes the senescence barrier and is a decisive step to malignant transformation.

and a concomitant loss of growth arrest. Thus, high-grade IEN appears to indicate the phase of which the senescence barrier is ruptured.

In conclusion, our data provide evidence for an oncogene-induced senescence in the serrated route to colorectal cancer with *BRAF* mutation. Upregulation of $p16^{Ink4a}$ expression appears to be a useful indicator of induction of senescence in *BRAF*-mutated serrated lesions. Loss of $p16^{Ink4a}$ expression is found during malignant transformation and is caused mainly by epigenetic silencing through aberrant methylation of the *CDKN2A* promoter.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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