

Expression of 11 β -hydroxysteroid dehydrogenase type 2 is deregulated in colon carcinoma

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Summary. Although the effects of glucocorticoids on proliferation, differentiation and apoptosis are well known, and steroid hormones have been identified to play a role in pathogenesis and the development of various cancers, limited data are available regarding the relationship between the local metabolism of glucocorticoids and colorectal adenocarcinoma (CRC) formation. Glucocorticoid metabolism is determined by 11 β -hydroxysteroid dehydrogenases type 1 and 2 (11HSD1, 11HSD2), which increase the local concentration of cortisol due to the reduction of cortisone, or decrease this concentration due to the oxidation of cortisol. The objective of this study was to evaluate the extent of 11HSD1 and 11HSD2 mRNA in pre-malignant colorectal polyps and in CRC. The specimens were retrieved from patients by endoscopic or surgical resection and the expression of 11HSD1 and 11HSD2 was measured by real-time PCR. The polyps were of the following histological types: hyperplastic polyps and adenomas with low- or high-grade dysplasia. The neoplastic tissue of CRC obtained during tumor surgery was also studied. It was found that 11HSD2 was not only downregulated in CRC but already in the early stages of neoplastic transformation (adenoma with low-grade dysplasia). In contrast, the level of 11HSD1 was significantly increased in CRC but not in pre-malignant polyps. The results demonstrate that the downregulation of 11HSD2 gene expression is a typical feature of the development of colorectal polypous lesions and their transformation into CRC.

Key words: 11 β -hydroxysteroid dehydrogenase, Colorectal polyp, Adenoma

Introduction

Colorectal adenocarcinoma (CRC) is one of the leading causes of cancer death worldwide. CRC appears to be the result of the progressive transformation of colonic epithelium into neoplastic tissue. The wild-type genes that act as suppressors of carcinoma cell growth are affected by *in vivo*-derived mutation resulting in the loss of their function (Fearon and Vogelstein, 1990). This neoplastic condition leads to the hyperproliferation of colonic crypts accompanied by a delay or inhibition of cellular differentiation and apoptosis and progresses through a series of stages, including the formation of adenomas that can be transformed into malignant tumors (Michor et al., 2005).

Enterocyte proliferation, cell cycle progression, differentiation and apoptosis are under the control of several regulatory pathways, especially the Wnt/ β -catenin/Tcf signaling pathway and its downstream effectors, and thus the deregulation of the Wnt pathway plays an important role in tumor formation (Oving and Clevers, 2002). Recent data indicate that the initiation of colorectal carcinogenesis is mostly linked to an abnormal activation of the canonical Wnt signaling pathway (Van Den Brink and Offerhaus, 2007; Polakis, 2007). Although most sporadic CRCs develop through the adenoma-carcinoma sequence associated with activation of Wnt signaling pathway described in the classic model of Vogelstein (Fearon and Vogelstein, 1990), a distinct group of CRCs could arise from

different heterogeneous precursor lesions (hyperplastic polyps and serrated adenomas) that follow a distinct sequence of events from polyps to CRC, the so-called serrated neoplasia pathway (Leggett and Whitehall, 2010). The published results of Wnt signaling activation in this pathway are conflicting. Some studies detected such activation (Yachida et al., 2009), but others have suggested that Wnt pathway activation is unlikely to contribute to tumorigenesis (Fu et al., 2011; Fujita et al., 2011).

The process of cell growth is also regulated by glucocorticoids that act as stimulators or inhibitors of cell proliferation (Dickmeis and Foulkes, 2011), depending on the cell type and concentration used (Maattem et al., 2007). The pro-apoptotic actions of glucocorticoids have been observed in a number of cancers, but glucocorticoids can also induce resistance to cell death in others (Herr et al., 2007; Maattem et al., 2007; Dickmeis and Foulkes, 2011). Although the role of glucocorticoid hormones in colorectal carcinogenesis is not well understood, the presence of glucocorticoid receptors in 50-80% of samples of CRC (Brentani et al., 1993; Theocharis et al., 2003) and in human colon carcinoma cell lines (Berrada et al., 1990; Meyer and Schmidt, 1995) indicates their possible significance. In addition, colorectal cancer cell lines and primary tumors express steroidogenic enzymes and produce a detectable level of cortisol (Sidler et al., 2011).

Glucocorticoid bioavailability and its effect on the target tissues depends not only on the number of glucocorticoid receptors and the plasma concentration of the hormone, but also on the local metabolism of glucocorticoids, which is determined by the enzyme 11 β -hydroxysteroid dehydrogenase (11HSD). This enzyme exists in two types; type 1 (11HSD1) operates *in vivo* as a reductase that converts biologically inactive cortisone into active cortisol, whereas type 2 (11HSD2) is an exclusive oxidase that converts cortisol into inactive cortisone (Draper and Stewart, 2005). In the human colon, 11HSD1 is located predominantly in subepithelial structures, whereas 11HSD2 is only found in epithelial cells (Whorwood et al., 1994). Although aberrations of 11HSD1 and 11HSD2 expression have been identified in human CRC (Žbáňková et al., 2004; Zhang et al., 2009), the question of whether aberrant 11HSD expression is present during the transition from normal colon to adenoma and from adenoma to carcinoma remains unknown. Therefore, the objective of this study was to evaluate the extent of 11HSD1 and 11HSD2 expression in different stages of malignant transformation of colonic epithelium and to assess the relationship between 11HSD expression and the degree of Wnt pathway activation, estimated as the expression of axin 2. Axin 2 functions as a negative regulator of Wnt pathway that is upregulated in human tumors associated with Wnt signaling but not in other tumor types, and thus represents a sensitive marker of Wnt pathway activation (Lustig et al., 2002).

Materials and methods

Patients and tissue samples

Specimens were retrieved from 10 patients who underwent surgical resection of colorectal cancer and 64 patients who underwent endoscopic polypectomy, respectively. The characteristics of the patients are given in Table 1. Representative samples of resected CRC were obtained from the central portion of the tumors, whereas the samples of control tissue were from the macroscopically normal areas of colonic epithelium at the margin of the resection. The stage of the tumors according to the American Joint Committee on Cancer (AJCC) was IIA in all cases. The resected specimens were processed for histological examination and all tumors were identified as colorectal adenocarcinomas. Well-differentiated carcinomas (grade 1) were observed in 10%, carcinomas of moderate differentiation (grade 2) in 70% and poorly differentiated carcinomas (grade 3) in 20% of samples. In the case of polypectomy, paired polyps (upper part) and biopsies from the normal rectosigmoid mucosa were analyzed. The specimens were evaluated by histological type and classified as hyperplastic polyps (HYP), adenomas with low grade dysplasia (LGA), and adenomas with high grade dysplasia (HGA) (Table 1). Patients who had been treated with chemotherapy or radiotherapy were excluded from this study. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University, Prague and all patients gave informed consent to participate in the study.

RNA extraction and quantitative real-time PCR from bioptic samples/polyps

Endoscopically obtained samples of polyp tissue and normal mucosa were homogenized using MagnaLyser Green Beads (Roche Diagnostics, Mannheim Germany) and the total RNA was isolated using a GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. 1.0 μ g of RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcriptase Kit and random hexamers

Table 1. Age and gender data of the patients.

	HYP	LGA	HGA	CRC
Age (years)	69 (61-76)	68 (51-93)	67 (37-86)	80 (6-96)
Gender (M/F)	5/3	14/16	16/10	3/7

Ages of patients are expressed as medians and ranges in parentheses; numbers of patients are given separately for men (M) and women (F). HYP, hyperplastic polyps; LGA, adenoma with low-grade dysplasia; HGA, adenoma with high-grade dysplasia; CRC, colorectal adenocarcinoma

(both Life Technologies, Carlsbad, CA, USA). The resulting cDNAs were analyzed by real-time PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Master Mix and TaqMan Assays (Life Technologies) specific to human 11HSD1 (cat.no. Hs00194153_m1), 11HSD2 (cat.no. Hs00388669_m1), axin 2 (cat.no. Hs00610344_m1), and the housekeeping gene TATA box-binding protein, TBP (cat.no. 4326322E-1010010). A single PCR reaction was performed in a final volume of 50 μ l using target gene probes labeled with FAM/TAMRA in conjunction with a TBP probe (VIC/MGB). Expression values were obtained from cycle threshold values detected by the Applied Biosystems analysis software and the calibration curve created by 10-fold dilutions of the mixed cDNA sample. The genes of interest were normalized to TBP.

Laser microdissection of neoplastic cells in colorectal cancer, RNA extraction and quantitative real-time PCR

Representative samples of the neoplastic cells of the CRC and the matched normal crypts of the tumor margins were dissected using laser microdissection (LMD) as previously described with some modifications (Švec et al., 2010). Briefly, the samples of resected CRC and the surrounding normal-looking colonic tissues were cut in a cryostat (Leica Microsystems CM 3000, Wetzlar, Germany) at -18°C . Frozen 8- μm -thick sections were mounted onto slides coated with polyethylene naphthalate membrane (Leica Microsystems), the slides were fixed with 95% ethanol, stained with 4% cresyl violet acetate and washed three times in 95% ethanol. Tumor cells and normal crypts of the margin of the resections were dissected using the LMD6000 Laser Microdissection System (Leica) and captured into caps of the microcentrifuge tubes. Microdissected tissues were centrifuged in 75 μ l RLT buffer (Qiagen, Hilden, Germany) and stored at -80°C until RNA isolation. The total RNA was isolated using an RNeasy Micro Kit (Qiagen, Hilden, Germany) and the RNA samples in the nanogram range were reverse-transcribed to cDNA with Enhanced Avian Reverse Transcriptase (Sigma-Aldrich) and random hexamers. Because the RNA yield of 11HSD1, 11HSD2 and axin2 transcripts was low, an aliquot of the cDNA sample was amplified with TaqMan PreAmp Master Mix Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting cDNA samples were analyzed by real-time PCR as mentioned above.

Immunohistochemistry of 11HSD1 and 11HSD2

For immunohistochemical staining, 5- μm sections were cut from formalin-fixed and paraffin-embedded tissue, dewaxed in xylene and rehydrated. The endogenous peroxidase activity was inhibited by 0.3% hydrogen peroxide in methanol for 30 min. To unmask

the antigen sites, the sections were treated with Target Retrieval Solution (Dako, Glostrup, Denmark). The primary anti-11HSD1 polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) was applied at 1:200 dilution for 1h. For detection of 11HSD2, rabbit polyclonal antibody (Proteintech, Chicago, IL, USA) was diluted 1:100 and incubated with the sections overnight at 4°C . The bound primary antibody was detected using EnVision+ System (Dako, Glostrup, Denmark) applied for 30 min and the brown color reaction was developed with DAB substrate for 5 min. Subsequently, the sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. Normal human liver and kidney samples were used as positive controls for 11HSD1 and 11HSD2, respectively. Sections incubated without primary antibody represented the negative controls. The sections were examined and fotodocumented using Nikon Eclipse E400 microscope with DS-Fi1 digital camera.

Statistical analysis

Data are presented as the median, the 25th and 75th percentile and the minimum-maximum range. Nonparametric statistical analysis was performed with the software Statistica 6.1 (StatSoft, Tulsa, OK). A comparison of mRNA levels between the tumor cells of colorectal carcinomas and the surrounding normal epithelial cells was made with the Mann-Whitney test. The polypoid lesions of low- and high-grade dysplasia and hyperplastic polyps were compared with a Kruskal-Wallis analysis of variance and Spearman's rank correlation test was used to evaluate a statistically significant correlation between the expression levels of 11HSDs and axin 2. A P value smaller than 0.05 was considered statistically significant.

Results

Relationship between histological types of polyps and expression of 11HSD1 and 11HSD2 mRNA

Histological analysis demonstrated the presence of three types of polypous lesions - hyperplastic polyps (HYP; n=8), adenomas with low-grade dysplasia (LGA; n=30), and adenomas with high-grade dysplasia (HGA; n=28). All polypous lesions and the corresponding biopsies of normal mucosa constitutively expressed both 11HSD1 and 11HSD2 (Fig. 1A,B). Compared with the biopsies of the normal mucosa of the same patients, the expression of 11HSD2 was significantly downregulated in both adenomas with low- and high-grade dysplasia (Fig. 1B). The qRT-PCR analysis showed a similar tendency in hyperplastic polyps, although the 11HSD2 gene did not reach statistical significance (Fig. 1B). In contrast, 11HSD1 expression was significantly downregulated only in the polyps of the HGA group (Fig. 1A).

One of the first events in conventional CRC

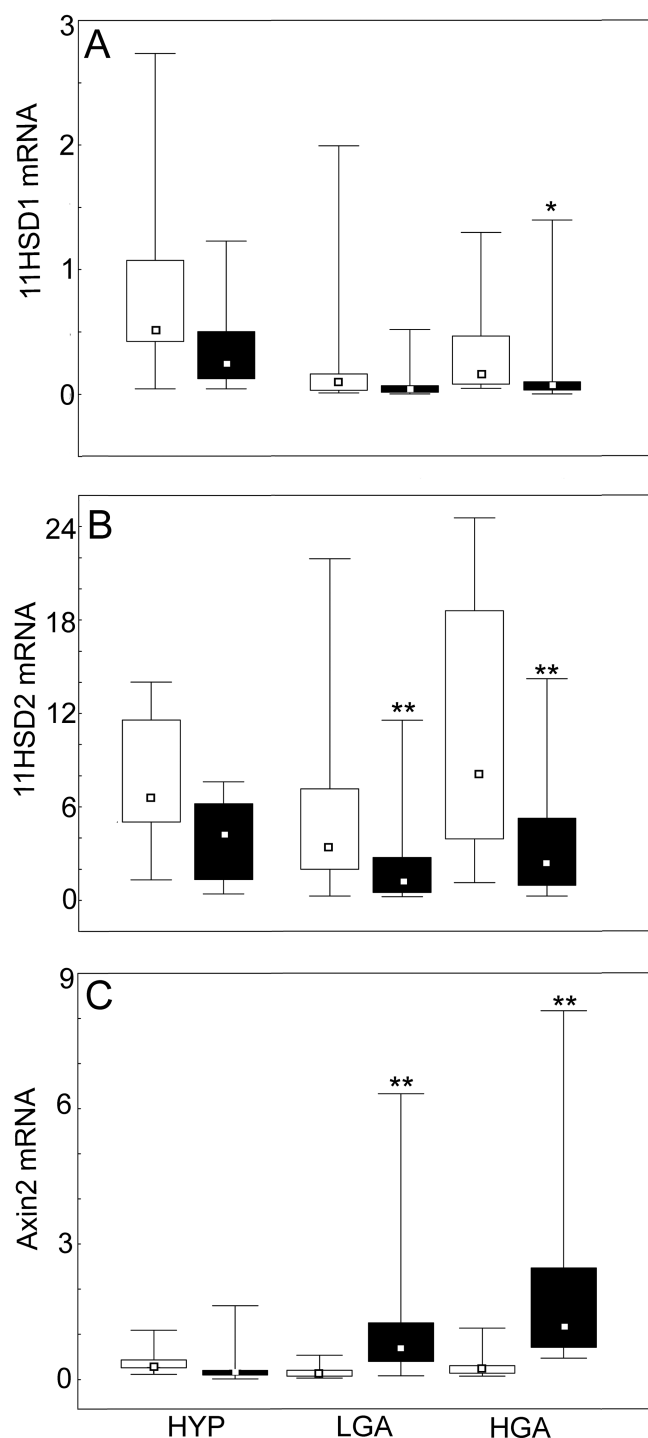


Fig. 1. Real-time PCR of 11HSD1 (A), 11HSD2 (B) and axin 2 (C) using RNA isolated from hyperplastic polyps (HYP, n=8), low-grade (LGA, n=30), and high-grade adenomas (HGA, n=26). Open bars, biopsies from the normal rectosigmoidal mucosa of patients undergoing polypectomy; filled bars, polypous lesions. Levels of gene of interest mRNAs were normalized to TATA box-binding protein gene and the data are given as median values, 25th and 75th percentiles and min./max. ranges. Significant difference between polyps and matched biopsies: * P<0.05, ** P<0.01

formation is usually the abnormal activation of the Wnt pathway, which is associated with the upregulation of axin 2, a sensitive marker of Wnt signaling activation (Lustig et al., 2002). As shown in Fig. 1C, axin 2 mRNA was significantly increased in both adenomas with low- and high-grade dysplasia. To investigate if any correlation exists between the expression profile of 11HSDs and Wnt activation, the data were analyzed using Spearman's rank correlation test. The test revealed a statistically significant positive correlation between the expression of 11HSD2 mRNA and axin 2 mRNA in LGA (RS=0.72, P<0.001) while in the HGA, the transcript levels did not correlate (RS=0.35, P=0.097). Neither was a correlation found between the axin 2 and 11HSD1 transcripts in LGA and HGA groups. We also evaluated the expression of axin 2 in hyperplastic polyps and did not find, in comparison to adenomas with low- and high-grade dysplasia, any upregulation of axin 2 mRNA (Fig. 1C).

Relationship between the expression of 11HSD1 and 11HSD2 mRNA in neoplastic cells and crypts of normal mucosa of patients with CRC

The specificity of 11HSD1 and 11HSD2 expression in polypous lesions is influenced by stromal cell contamination of the samples. To overcome this problem in CRC samples, the neoplastic cells and the non-neoplastic crypts of resected samples were dissected by LMD and the expression of 11HSD1 and 11HSD2 genes quantified. The transcript of 11HSD2 was found in both tissues and in accordance with the above-mentioned data, 11HSD2 was significantly downregulated in neoplastic epithelium (Fig. 2B). In contrast, the expression of 11HSD1 was only marginally detected in some specimens of the control colonic epithelium of patients with CRC, but the level of 11HSD1 mRNA was significantly increased in neoplastic epithelium (Fig. 2A). Axin 2 was significantly upregulated in neoplastic epithelium (Fig. 2C).

Immunolocalisation of 11HSD1 and 11HSD2 during colon tumorigenesis

We used rabbit polyclonal antibodies to detect the expression of 11HSD1 and 11HSD2 proteins in colon samples with different stages of neoplastic transformation from normal epithelium to adenocarcinoma and in control tissues. The results of immunohistochemical staining are presented in Fig. 3 (A-L). In normal liver tissue, used as a positive control, the expression of 11HSD1 was diffuse with granular cytoplasmic staining accentuated in hepatocytes near the central vein (Fig. 3A). In normal colon tissue, cytoplasmic 11HSD1 immunoreactivity was observed in epithelial cells along the crypts with higher intensity in the luminal compartment. There was also cytoplasmic and nuclear positivity present in smooth muscle and stromal cells (Fig. 3C). In hyperplastic polyps, 11HSD1

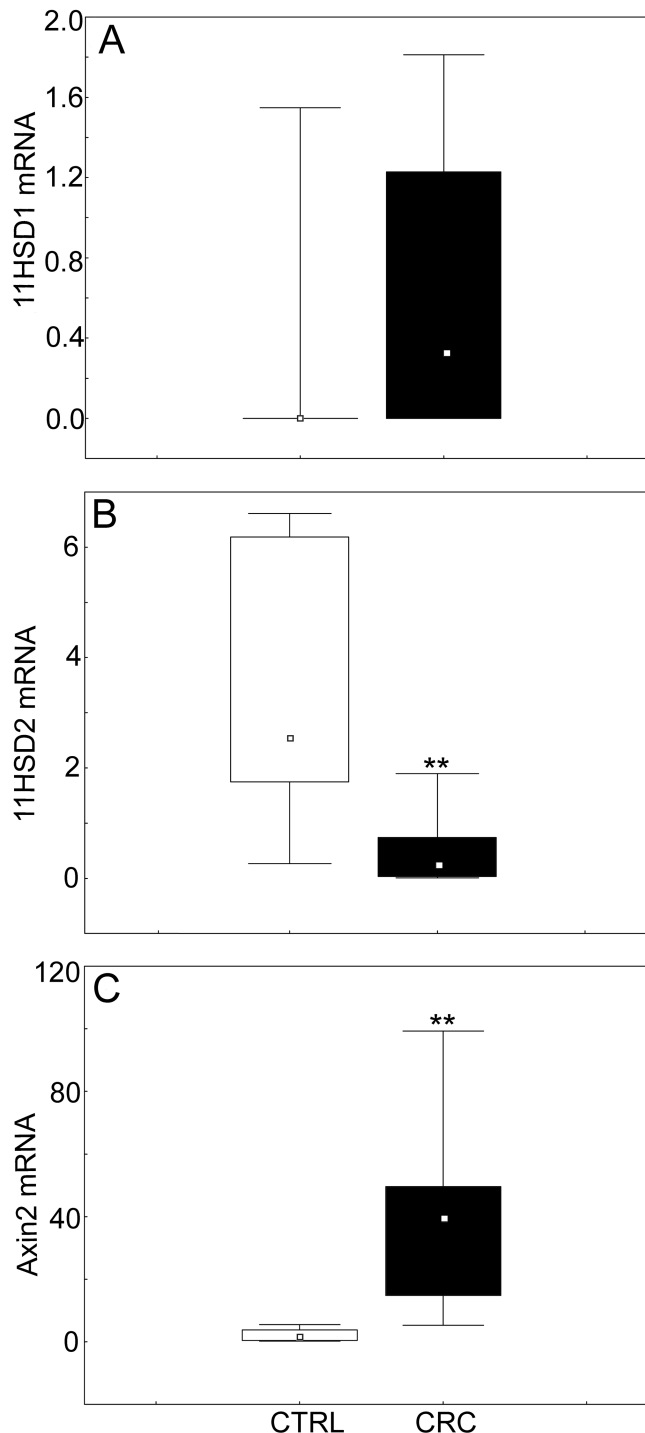


Fig. 2. Real-time PCR of 11HSD1 (A), 11HSD2 (B) and axin 2 (C) using RNA isolated from neoplastic cells (CRC) and normal epithelium (CTRL) of patients with colorectal cancer (n=10). 11HSD1 signal was detected only in 2 samples of normal epithelium. For further details, see Fig. 1. Significance relative to the matched normal epithelium: ** $P < 0.01$

staining retained a similar pattern to normal colon epithelium, whereas adenomatous crypts displayed irregular distribution of 11HSD1 staining (Fig. 3G). In some adenocarcinoma samples we observed strong cytoplasmic immunopositivity (Fig. 3K).

Kidney tissue sections were used as positive control for 11HSD2 immunodetection with strong expression observed predominantly in distal convoluted tubules and collecting ducts (Fig. 3B). In normal colon tissue, 11HSD2 expression was present only in epithelial cells, where the increasing gradient along the longitudinal axis of the crypt was demonstrated with maximum positivity in the superficial epithelium (Fig. 3D). This pattern of expression was also shown in hyperplastic crypts, although in adenomatous tissue irregular staining varied among the crypts with decreasing intensity in high-grade adenomas (Fig. 3J). Only weak immunoreactivity for 11HSD2 was detected in the majority of adenocarcinoma samples (Fig. 3L).

Discussion

This study demonstrated a downregulation of 11HSD2 expression in all investigated neoplastic tissues that clearly distinguishes them from the nontransformed cells. The finding of 11HSD2 mRNA being already downregulated in adenomas with low-grade dysplasia indicates that the gene expression of 11HSD2 changes very early in colorectal carcinogenesis and persists during the sequence normal epithelium - low-grade dysplastic adenoma - high-grade dysplastic adenoma - carcinoma. In this context, the downregulation of 11HSD2 mRNA and 11 β -oxidase activity was found in colorectal carcinoma by our group (Žbáňková et al., 2004) but not by others (Zhang et al., 2009). The downregulation of 11HSD2 has also been reported in human cortisol and aldosterone secreting adrenal adenomas (Albertin et al., 2002; Mazzocchi et al., 2002), in clear cell type of renal cell carcinomas (Yakirevich et al., 2008) and in some endometrial tumors (Manolis et al., 2006).

The intestinal mucosa is able to synthesize glucocorticoids due to steroidogenic enzymes in intestinal crypts (Cima et al., 2004). In addition, the capacity to produce cortisol is also retained in human colon cancer cells (Sidler et al., 2011). As CRC cells are often immunogenic and can stimulate tumor lymphocytes (Camus et al., 2009), and intestinal glucocorticoid synthesis is induced upon the activation of immune cells (Cima et al., 2004), the downregulation of 11HSD2 expression in neoplastic cells might modulate the glucocorticoid signals important for the regulation of local intestinal immune cells. The signal of glucocorticoids produced in situ exhibits both an inhibitory and costimulatory role on intestinal T cell activation, depending on the mode of activation (Cima et al., 2004), thus the changed expression of 11HSD2 in neoplastic tissue might be considered to be a factor related to tumor immune surveillance, development and

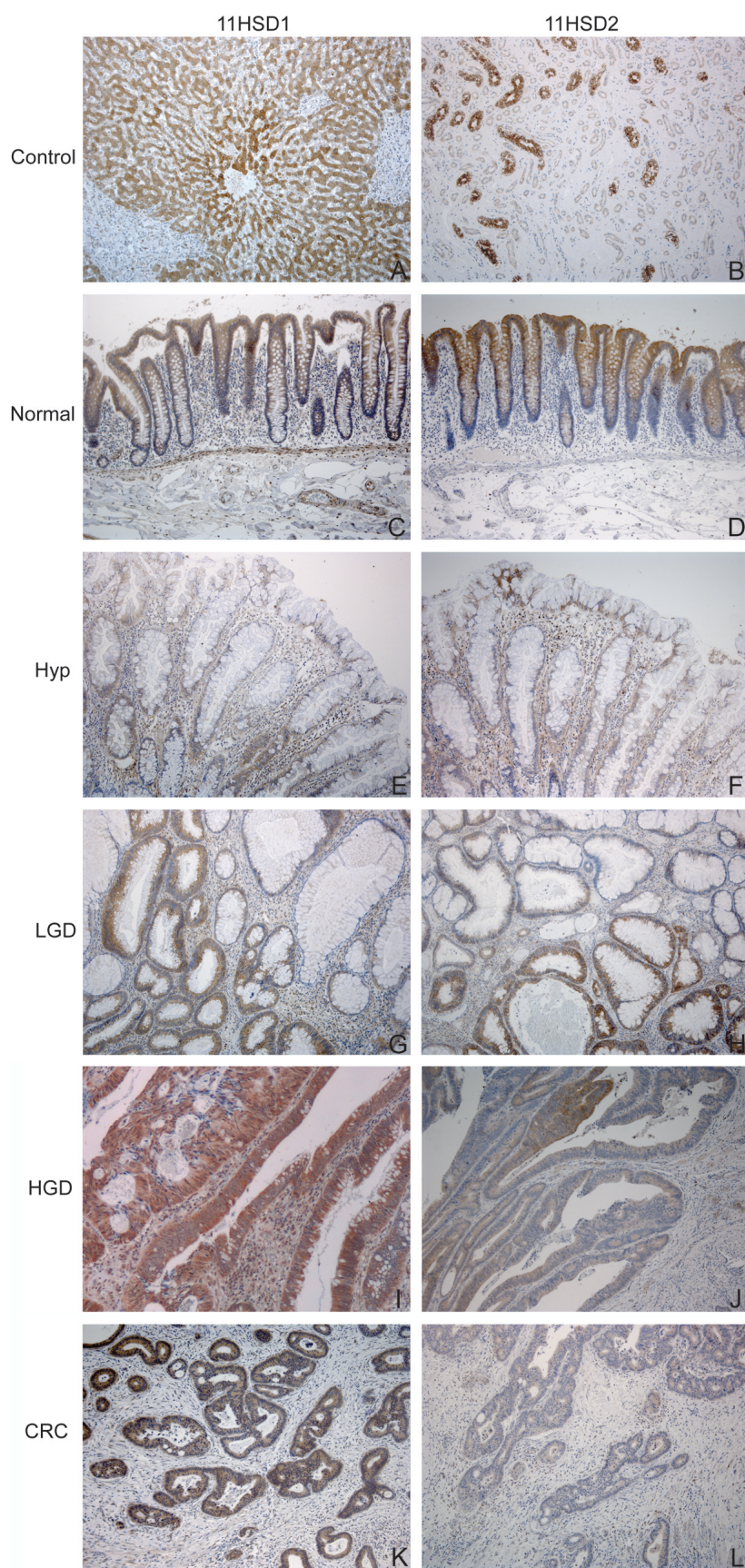


Fig. 3. Immunohistochemical staining of 11HSD1 and 11HSD2 in human colon tissue with different stages of neoplastic transformation and in positive control samples. Control liver and kidney tissue (**A and B**); normal colon mucosa (**C and D**); hyperplastic polyp, HYP, (**E and F**); adenoma with low-grade dysplasia, LGD, (**G and H**); adenoma with high-grade dysplasia HGD, (**I and J**); adenocarcinoma, CRC, (**K and L**). x 100

growth. The critical contribution of in-situ-produced intestinal glucocorticoids to the immune homeostasis of intestinal mucosa during the development of intestinal inflammation has been recently proved (Coste et al., 2007; Noti et al., 2010).

As for 11HSD1, there are few studies analysing its expression and role in tumorigenesis, often with conflicting results. While in certain types of malignant tumors, like oropharyngeal (Gronau et al., 2002) or breast cancer (Lu et al., 2011), 11HSD1 expression was found to be downregulated, increased expression was noted in colon cancer specimens (Storkson et al., 2012) and was shown to contribute to glucocorticoid sensitivity in leukemia cells (Sai et al., 2009). In our study this enzyme was found in the neoplastic tissue of CRC but was nearly absent in the normal-looking colonic epithelium of the same patients when laser-microdissected samples were analyzed. In contrast, its expression was found in adenomas and hyperplastic polyps and matched control biopsies using both qPCR and immunohistochemistry and reached statistically significant upregulation in adenocarcinoma samples. Tumor-associated immune cells could be another source of 11HSD1 expression, as we observed a stronger 11HSD1 signal in specimens that have been more extensively infiltrated with leukocytes (data not shown), and intestinal immune cells are a well known source of this enzyme (Ergang et al., 2011). Nevertheless, further functional studies are needed to assess whether 11HSD1 may contribute to cancer progression.

In summary, the decreased expression of 11HSD2 in different stages of neoplastic tissue indicates a possible role of this change in glucocorticoid signaling during the transformation of colorectal epithelium from the early stages of development. This conclusion is supported by the significant positive correlation between axin 2 (a marker of Wnt activation which is associated with neoplastic cells of colorectal epithelium) and 11HSD2 expression in low-grade dysplasia. The downregulation of 11HSD2 expression during the transformation of colorectal epithelium might participate in the control of tumor-associated immune cells.

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