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Two initiation sites of early detection of colon cancer revealed by localization of pERK1/2 in the nuclei or in aggregates at the perinuclear region of the tumor cells

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ABSTRACT

We have used human specimens and antibodies to pERK1/2 to detect early development of colon cancer using indirect immunocytochemistry. Two distinct sites were stained; one at the tip of the colon crypts and the other in the stromal tissue associated with the colonic tissue. These foci represent early stages of colon cancer initiation sites as established by enhanced Kirsten Rat Sarcoma Virus (KRAS) and the lack of p53 staining. The enhanced KRAS coincides with the initiation of tumor growth revealed by pERK1/2, both in the tip of the colon crypts, as well as in the stromal initiation site of the colon tumors. Foci of pERK1/2 staining were also detected in 50% of stromal tissue and tips of colon crypts, which were classified as normal tissues, adjacent to the malignant tissue according to general morphology. However, in colon specimens, where no malignancy was observed, no accumulation of pERK1/2 was observed. The staining of pERK1/2 at the stromal foci of the apparently non-malignant tissue appeared as aggregates in the perinuclear region, while in the colon epithelium it appeared in the cell nuclei. In low-grade colon cancer that was still free of induced mutated p53, staining of pERK1/2 was prominent in the cell nuclei, both in the stroma tissue and the tip of the colon crypts. In the intermediate stage, that exhibited significant p53 staining, only a fraction of p53-free tumor cells was labeled with pERK1/2 antibody, while in high-grade tumors, all cells of tumors were labeled with antibodies to p53, but not with antibodies to pERK1/2. We suggest that the down regulation in pERK1/2 labeling is due to the mitogenic capacity of the tumor cells, which are shifted from being driven by nuclear pERK1/2 to mutated p53 expression. We also found that the cytoplasm of low grade tumors was positive for epiregulin, while this labeling decreased in high-grade tumors. We found that the tumors arising from the stroma demonstrated poor structural differentiation, while the tumors initiating from the epithelial cells of the colon demonstrated high structural differentiation. We conclude that pERK1/2 is a sensitive marker of early colon cancer, which disappears at later stages of cancer development. Moreover, pERK1/2 staining can distinguish between tumor cells originating from the tip of the colon crypts and those developing in the stroma, which is present in the close vicinity to colon epithelial tissue, and thus can assist in selecting the appropriate therapy.

Introduction

Colorectal cancer is the second leading cause of cancer deaths in Western countries. Despite more than two decades of research into the molecular genetics of colon cancer, there is still a lack of prognostic and productive molecular biomarkers with proven utility in this setting (Baba et al., 2000; Matsumoto et al., 2011; Manzano and Perez-Segura, 2012). It has been suggested that balance between MAPK pathways could be critical for presenting or promoting growth in a variety of cancer cell lines, including colon cancer cell lines (Baba et al., 2000). In additional studies, the essential role of mutation of Kirsten Rat Sarcoma Virus (KRAS) and p53 in colorectal cancer was established (Ueda et al., 2003; Klump et al., 2004; Khambata-Ford et al., 2007). It was discovered that loss of epigenetic control of synucleins serves as a molecular indicator of metastasis in a wide range of human cancers, including colon cancer (Liu et al., 2005, 2010). It was also speculated that UCP2 altered suppression, oxidative stress and NF-κB activation may be related

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to successive events in cancer development, including colon cancer (Derdák et al., 2006). Moreover expression of epiregulin and KRAS mutation was found valuable in predicting control of metastatic colorectal cancer, using specific drugs as Cetaxumab (Ueda et al., 2003; Li et al., 2010). High expression of ERBB1, ERBB2 and ERBB3, but not ERBB4, was found in colon cancer cell lines and could help in the selection of the appropriate chemotherapy (Liu et al., 2005; Wu et al., 2009). High serum and tissue levels of amphiregulin and high tissue levels of epiregulin have been proposed as predictors of poor prognosis in patients with colorectal carcinoma (Nishimura et al., 2008; Jacobs et al., 2009; Wu et al., 2009; Li et al., 2010).

It is well established that the interaction of EGF ligands of the EGF family, epiregulin and ampiregulin, with the appropriate receptors (ERBB) will lead to phosphorylation of the receptor molecule (Bae and Schlessinger, 2010), which will eventually phosphorylate MAK and ERK1/2, leading to their translocation into the nuclei of the cancer cells (Jaaro et al., 1997; Chuderland et al., 2008; Yao and Seger, 2009; Keshet and Seger, 2010; Plotnikov et al., 2011).

The intestinal renewal system is tightly controlled and depends on the spatial organization of signals that emanate from supportive mesenchymal cells, as well as from differentiated epithelial progeny. Recent evidence suggests that intestinal cancers may still contain a hierarchical organization, with cancer stem cells (CSCs) at the apex (Vermeulen et al., 2008; Amsterdam et al., 2012a). From the seminal studies of Fearon and Vogelstein (1990) and Amsterdam et al. (2012b) it is clear that CSC develops as a stepwise accumulation of genetic hits in specific genes and pathways. The CSC theory refines this model further and suggests that the actual tumorigenic capacity of individual cancer cells may be influenced by homeostatic signals derived from their microenvironment. These findings are especially exciting in light of recent developments that have increased our comprehension of the regulatory mechanisms that control individual stem cells (ISCs), and have resulted in new tools to identify and localize ISCs (Amsterdam et al., 2012b,c). Although we clearly do not fully grasp the complete spectrum of signals and interactions at this point, our understanding of normal crypt homeostasis and the identification of markers that define ISCs are providing intriguing insights into the organization of intestinal cancers (Fearon and Vogelstein, 1990; Medema and Vermeulen, 2011; Amsterdam et al., 2012c).

In recent studies it was shown how RAS activation causes aberrant nuclear localization of phosphorylated MAPK and ERK to drive neoplastic transformation in colorectal tumors and human colon cancer cells. However, in these reports there was no follow-up of the appearance of nuclear pERK1/2 in the different stages of this disease and there were no studies on the correlation between the appearance of pERK in relationship with the appearance of mutated p53 (Duhamel et al., 2012; Zhao et al., 2011).

In the present study we demonstrate that pERK1/2 could serve as an early marker for the development of colon cancer and may be present preferentially in colon cancer stem cells. Moreover, their distribution in the colonic tissue and associated stromal tissue could explain multiple sites of cancer development that could be recognized by the presence of pERK1/2, which would disappear at later stages of colon cancer development, coinciding with the appearance of mutated p53.

Materials and methods

Human tissue samples

We analyzed normal tissues of colon: 4 μm sections of formalin fixed and paraffin embedded tissues that were removed adjacent to the tumor (11 samples) and were classified as normal tissue according to general morphology of sections stained with hematoxylin and eosin (H&E). In addition, we analyzed 3 colon specimens with no history of cancer. We also analyzed tissues of low grade tumors (stage I) (12 samples), intermediate (stage II) (11 samples) and high grade tumors (stages III and IV) (13 samples). 4 μm thick sections were stained by the indirect immunocytochemistry method (Ginath et al., 2001; Singer et al., 2005; O'Neill et al., 2005; Zeren et al., 2008; Amsterdam et al., 2011, 2012a, 2012b, 2012c) with anti-pERK1/2 or anti-p53 or anti-epiregulin or anti-KRAS antibodies followed by staining with hematoxylin for 90 s. It should be noted that the number of samples referred to above indicates the number of patients from whom the tissue samples were collected. All biopsies were obtained with permission of the Helsinki Committees of the Wolfson Hospital in Holon and Kaplan Hospital in Rehovot, Israel, receiving informed consent.

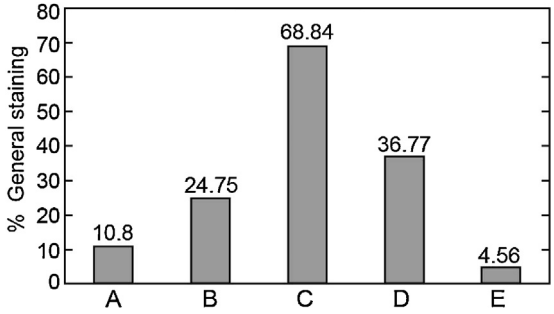

Fig. 1. Percentage of immunolabeled cells with pERK1/2 in the development of colon cancer. (A) Presumptive normal. Both perinuclear and nuclear predominated. (B) Low grade; perinuclear immunostaining predominated. (C) Low grade; nuclear predominated. (D) Intermediate grade. Mainly nuclear immunostaining predominated. (E) High grade. Mainly nuclear predominated.

Reagents

Primary antibodies used

(1) Monoclonal antibodies to p53 (mouse clone 421) that were kindly donated by Prof Moshe Oren from the Weizmann Institute of Science.
(2) Antibodies to pERK1/2 were mouse monoclonal antibodies that recognize only the phosphorylated form of ERK 1/2 and not the non-phosphorylated form, using a dilution 1:200 (M8159; Sigma–Aldrich, St. Louis, MO, USA).
(3) Antibodies to epiregulin were anti-human recombinant epiregulin and were affinity purified (1195-EP, R & D Systems, MN, USA).
(4) Rabbit polyclonal antibodies to KRAS, purified (Acris Antibodies, San Diego, CA, USA). Following incubation with the first antibodies there was incubation with specific secondary antibodies against the first ones conjugated to peroxidase (N-Histofine, Tokyo, Japan) and the staining of the slides was performed as described elsewhere (Ginath et al., 2001; Singer et al., 2005; O'Neill et al., 2005; Zeren et al., 2008; Amsterdam et al., 2011, 2011b, 2012a).

Specificity of the staining

Negative controls were performed by staining the sections with the appropriate IgG fractions at 10 μg/ml. Positive controls for p53, PERK and KRAS were as described by Amsterdam et al. (2011, 2011b, 2012b). Positive controls to KRAS antibodies were also provided by Acris Company demonstrating human prostate carcinoma stained specifically with anti-KRAS antibodies.