Use of multiple biomarkers for the localization and characterization of colon cancer stem cells by indirect immunocytochemistry

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Abstract. In this study, we used LGR5, γ-synuclein, p53, KRAS and epiregulin antibodies to localize stem cells by indirect immunocytochemistry in paraffin sections of normal and cancerous colon tissues. In the normal colon tissue, no staining of cells with LGR5, γ-synuclein, p53 and KRAS antibodies was observed, apart from a few scattered cells in between the colon villi that were faintly stained with antibodies to LGR5. Staining of highly differentiated cancer tissue with LGR5 antibodies revealed single cells or clusters of up to 4 cells in the interior space of the carcinoma cell layers. Staining of poorly differentiated cancer tissues (stage I-IV) revealed 9-81 clustered stem cells. The number of clustered stem cells increased significantly with the tumor stage, when comparing stage II to stage IV (p<0.0048). Occasionally, the clustered stem cells appeared in the interphase between the colon stroma and the tumor tissue. Surprisingly, antibodies to p53 clearly stained the clusters of stem cells both in the nuclei and the cytoplasm. The staining of the nuclei of other cells in the undifferentiated tumors was in general weaker, and no staining was found in the cytoplasm. Antibodies to γ-synuclein heavily stained the endothelial cells of the blood vessels and some other scattered cells in the highly differentiated tumors. Antibodies to γ-synuclein heavily stained the stem cells in both the cytoplasm and the nuclei of poorly differentiated tumors. Antibodies to KRAS stained the cytoplasm and the nuclei of stem cells in poorly differentiated tumors and also stained the cytoplasm of some scattered cells. Antibodies to epiregulin stained the cytoplasm of normal colon tissue cells in the crypt-villus axis. The antibodies weakly stained the highly differentiated tumor cells and moderately stained the moderately differentiated tumor cells. Of note, the antibodies intensively stained the clustered stem cells of the poorly differentiated tumor cells. These antibodies also clearly stained the clustered stem cells of poorly differentiated tumors but were not specific as they clearly stained cells in the crypt-villus axis of the normal colon wall. Our results show that LGR5 antibodies can serve as a reliable marker for colon cancer stem cells. Once the colon stem cells are identified, the targeting of specific drugs to kill these cells should be attempted in the future in order to cure this disease. Moreover, the fact that we did not find any stained cells with antibodies to LGR5 in normal tissues apart from a few scattered cells, suggests that the normal colon stem cells differ from the tumor stem cells at least as regards the expression of this protein. In addition, antibodies to γ-synuclein, p53 and KRAS only stained the tumor stem cells and not the normal tissue. Thus, they can serve as multiple biomarkers for the localization of colon cancer stem cells by indirect immunofluorescence.

Introduction

Cancer cells in a tumor are heterogeneous with a subpopulation of unique neoplastic cells, having the capacity of self-renewal and differentiation into progenitor cells that in turn differentiate into all types of rapidly proliferating neoplastic cells within the same tumor, forming a tumor mass (1,2). These self-renewing cancer cells are rare, but long-lived, and termed cancer stem cells (CSCs). The existence of CSCs in a malignant tumor may explain the long-standing pathological observation that solid cancers are not homogeneous in morphology and that multiple partially differentiated cancer cell populations in different stages of the same malignancy can co-exist within one patient (3-5). This may also be the reason for the failure of conventional anticancer therapies (6,7). A thorough understanding of CSCs is critical for the elucidation of the mechanisms of carcinogenesis and the search for curative anticancer therapy.

CSCs are thought to originate from mutant wild-type stem cells (8,9) and play critical roles in the development and maintenance of a malignant neoplasm (10). In colorectal cancer (CRC), the search for such a cancer-initiating stem cell has been intense for the past decades. In 2007, in a study on several intestinal WNT target genes, Barker et al discovered that the
leucine-rich-repeat-containing G protein-coupled receptor 5 (LGR5), i.e., G protein-coupled receptor 49 (GPR49), had a restricted expression in the crypt base of small and large intestines and was qualified as the stem cell marker for cells with intestinal differentiation (11-13). LGR5 is an orphan G protein with 7 transmembrane subunits, belonging to the glycoprotein hormone receptor super-family and expressed in skeletal muscle, placenta, spinal cord and brain tissue (14). This molecule was initially found for controlling specific embryonic tissue differentiation and growth (15). Subsequent studies from several laboratories have further demonstrated its expression in hepatocellular carcinoma (HCC), ovarian cancers, CRC and basal cell carcinoma (16-18). However, the underlying mechanisms for the involvement of LGR5 in carcinogenesis are poorly understood.

It is generally accepted that the WNT/β-catenin pathway plays a critical role in CRC tumorigenesis (19). Since Korinek et al (20) first reported the complete depletion of stem cells in the small intestine after the mutation of β-catenin and the Tcf4 interaction partner in mice, several studies have subsequently shown evidence of the important role of WNT signaling in stem cell maintenance and development (4,21-24). Yet, little is known about the correlation between LGR5 expression and the WNT/β-catenin pathway in CRC (25). There is also no report of the in situ localization of stem cells in colon cancer tissue, using antibodies to LGR5.

Synucleins are a family of small proteins consisting of 3 known members, synuclein α (SNCA), synuclein β (SNCB) and synuclein γ (SNCG). While synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, SNCA and SNCB have been specifically implicated in neurodegenerative diseases. SNCG, initially identified as a breast cancer-specific gene, is not clearly involved in neurodegenerative diseases but primarily involved in neoplastic diseases. SNCG overexpression in breast cancer cells stimulates proliferation, induces metastasis, promotes chromosomal instability, inhibits the mitotic checkpoint, and increases resistance to certain chemotherapeutic or anti-microtubule agents; however, the down-regulation of SNCG expression sensitizes breast cancer cells to anti-microtubule agent-induced cytotoxicity. Being identified as a breast cancer-specific gene, SNCG is aberrantly expressed in malignant breast cancer cells but not in the adjacent normal cells. So far, the abnormal expression of the SNCG protein has been demonstrated in f2 different malignant diseases, including ovarian, liver, esophageal, colon, gastric, lung, prostate, pancreatic, bladder and cervical cancers, as well as glial tumors. In these studies, the SNCG protein was abnormally expressed in a high percentage of tumor tissues but rarely expressed in tumor-matched non-neoplastic adjacent tissues.

The clinical relevance of SNCG expression in breast cancer prognosis has been confirmed in clinical follow-up studies. Patients with an SNCG-positive tumor have a significantly shorter disease-free survival and overall survival compared with those with no SNCG expression. However, the prognostic significance of SNCG in other cancers remains unknown (26).

It is known that the p53 tumor suppressor is the most frequent target for genetic alterations in human cancers and is mutated in almost 50% of all tumors. The inactivation of the p53 gene is a critical step in tumorigenesis. Following induction by a variety of cell stresses, such as DNA damage, hypoxia and the presence of activated oncogenes, p53 up-regulates a set of genes that can promote cell death and growth arrest, such as p21, GADD45, cyclin G and Bax. Recently, it was shown that p53 can repress the promoter activities of a number of anti-apoptotic genes and cell-cycle genes, such as survivin, cyclin B1, cdc2, cdc25 c, strathmin, Map4 and Bcl-2. Among the reported mutations, 75% are missense mutations, with 80% of them located in the DNA-binding domain of p53, and 30% of the mutations are in 5 hot-spot codons: 175, 245, 248, 273 and 282. Arginine residues (248 and 273) involved in the interaction of p53 with DNA, and arginines (175 and 282) stabilize the DNA-binding sequence. Wild-type p53 binds to promoters differently; for example, p53 activates the p21 promoter with higher affinity than the Bax promoter. Some p53 mutants are able to transactivate different genes, such as EGFR, MDR1, c-Myc, PCNA, IGF-2 or VEGF, providing growth-promoting phenotypes and drug resistance.

Mutation in the gene coding for p53 leads to overexpression of the protein and thus can be easily identified by techniques such as immunocytochemistry (27).

KRAS and BRAF mutations lead to the constitutive activation of EGFR signaling through the oncogenic Ras/Raf/Mek/Erk pathway. Currently, KRAS is the only potential biomarker for predicting the efficacy of anti-EGFR monoclonal antibodies (mAbs) in CRC. However, a recent report suggested that the use of cetuximab was associated with survival benefits among patients with p.G13D-mutated tumors. Furthermore, although the presence of mutated BRAF is one of the most powerful prognostic factors for advanced and recurrent CRC, it remains unknown whether patients with BRAF-mutated tumors experience a survival benefit from treatment with anti-EGFR mAb. Thus, the prognostic or predictive relevance of the KRAS and BRAF genotype in CRC remains controversial despite several investigations. Routine KRAS/BRAF screening of pathological specimens is required to promote the appropriate clinical use of anti-EGFR mAb and to determine malignant phenotypes in CRC. The significance of KRAS/BRAF mutations as predictive or prognostic biomarkers should be taken into consideration when selecting a KRAS/BRAF screening assay (28).

The epidermal growth factor (EGF) family of peptides encodes several proteins that can function as growth factors. The EGF-like peptides, such as epiregulin, bind and activate tyrosine kinase receptors that belong to the erbB family. The EGF-like peptides are overexpressed in a majority of human carcinomas as compared with their non-transformed counterparts. By using different approaches, it has been shown that several different EGF-like peptides function as autocrine growth factors in carcinoma cell lines of different histological origin. Direct evidence that the EGF-like growth factors might function as transforming genes has been provided by in vitro and in vivo studies. In particular, the development of different transgenic mouse lines in which EGF-like growth factors have been overexpressed by means of tissue-specific or non-specific promoters has provided invaluable information relating to their ability to function as dominantly transforming oncogenes. The cooperation of the EGF-like peptides with cellular proto-oncogenes in determining cell transformation has been demonstrated by using both in vitro and transgenic mouse systems. Taken together, these data strongly suggest that the EGF-like peptides are involved in the pathogenesis of human carcinomas, and that they might represent suitable targets for novel therapeutic approaches (29).
In the present study, we show that the vast majority of colon CSCs is embedded in clusters in poorly differentiated tumors, using LGR5 antibodies as a marker for these cells, while only a few stem cells are present in highly organized colon cancer tissue. No labeled cells with antibodies to LGR5 could be identified in the normal colon tissue, apart from a few scattered cells in between the colon villi.

Materials and methods

Materials. Polyclonal affinity purified antibodies to KRAS and LGR5 were obtained from Acris (Herford, Germany). N-histofine immunohistochemical reagent was obtained from Tokyo, Japan. The affinity purified antibody, anti-human epiregulin, was obtained from R&D Systems (Minneapolis, MN, USA). Anti-γ-synuclein, clone EP1539 Y rabbit monoclonal antibodies were obtained from Molsheim, France. Monoclonal antibodies

![Figure 1. Staining of sections of normal colon and tumor tissue with antibodies to LGR5. (A) No staining was evident using antibodies to LGR5 in the section through the villi-crypt axis of the healthy wall of the colon. Original magnification, x400. (B) Carcinoma tissue stained with antibodies to LGR5. Stem cells are stained in their membrane and cytoplasm as single or tiny clusters (arrows) in between the organized carcinoma cells. Original magnification, x400. (C) Poorly differentiated stage II tumor stained with antibodies to LGR5. A cluster of 45 cells is seen in the field stained in the membrane and in the cytoplasm (double arrows). Cells outside the cluster of stem cells remained unstained. In the upper part of the image are tumor cells and in the lower part are stromal cells of the colon tissue. Original magnification, x400. (D) A large cluster of 71 stem cells stained with antibodies to LGR5 in a poorly differentiated stage IV tumor. At the bottom left is the stroma tissue (St) and at the upper right are tumor cells of colon tissue (Tu). Original magnification, x 400.](image1)

![Figure 2. Statistical evaluation of the difference in size of clustered stem cells in highly differentiated stage II tumors, poorly differentiated stage II tumors and poorly differentiated stage IV tumors. The size of clustered highly differential (HD) colon cancer stem cells is 2±1 (mean ± SD) at stage II. The size of clustered stem cells in the poorly differentiated (PD) stage II tumors is much bigger, 25±14 (mean ± SD) and at stage IV is 61±15 (mean ± SD) Comparison of * to **; p<0.0002 and ** to ***; p<0.0048.](image2)
to p53 were a generous gift from Professor Moshe Oren from the Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel.

Methods. Paraffin blocks containing tissues of 3 normal colon and paraffin blocks containing 3 stage I-IV tumor samples at of highly differentiated, moderately differentiated and poorly differentiated cancers were cut at 4-mm thickness and stained with the various antibodies followed by secondary antibodies and staining with hematoxylin for 90 sec (30-33). For statistical quantification of the number of stem cells in the sections stained with LGR5 of each stage of the tumor and each degree of differentiation, the tissue sections were inspected under a Nikon microscope (Tokyo, Japan) and 4 fields of each patient tissue sample were photographed at a magnification of x400. The number of stem cells at each photograph was scored and the mean value ± SD of each stage of the tumor at the different stages of differentiation was calculated. Analysis of variance (ANOVA) was used to calculate the number of highly differentiated tumor stem cells in poorly differentiated stage II and poorly differentiated stage IV tumor tissues. ANOVA followed by a multiple comparison Student's t-test were performed. Calculations were performed using SPSS software (Student's t-test, version II; SPSS, Chicago, IL, USA). Values of p<0.05 were considered to indicate statistically significant differences.

Results

The LGR5 protein has been reported to be expressed in the brain (34), skeletal muscle (35), placenta (36) and human germ cells (37). Recently, it was characterized as a marker for stem cells in soft tissue sarcoma (38). In this study, we attempted to use
antibodies to LGR5 as a marker for stem cells in human colon cancer. In normal tissues no cell staining was evident (Fig. 1A) apart from a few scattered cells in between the colon villi (data not shown). In highly differentiated colon cancer (carcinomas) of stage II to IV, only occasional clusters of 2±1 stem cells (mean ± SD) were evident in between the organized carcinoma cell layers (Figs. 1B and 2). In contrast, in poorly differentiated stage II colon cancer cells, more often, larger clusters of stem cells of 25±14 (mean ± SD) were evident (Figs. 1C and 2). This number was significantly higher compared to that found among highly differentiated colon cancer cells (Figs. 1C and 2) (p<0.0002). In poorly differentiated stage IV cancers even bigger clusters of 65±12 (mean ± SD, p<0.00048) were evident among the cancer cells (Figs. 1D and 2). In highly differentiated tumors, staining with γ-synuclein antibodies was evident in the endothelial cells of the blood vessels and in some scattered tumor cells (Fig. 3A).

The incubation of the tissue sections of poorly differentiated cells (tumors stage II) with antibodies to γ-synuclein strongly stained the clusters of the stem cells in the cytoplasm as well as the nuclei of these cells. The clusters of stem cells were occasionally located between the stroma and the tumor tissue (Fig. 3B). The size of the clustered stem cells of stage IV of poorly differentiated cancer was even bigger than the size of the clustered stem cells at stage II of the cancerous tissue (Fig. 3C). Staining of the sections with antibodies to p53 clearly stained the nuclei as well as the cytoplasm of the stem cells. In other cancer cells only the nuclei were stained leaving the cytoplasm unstained. The staining of the nuclei of the other cells was, in general, weaker than in the nuclei of the stem cells (Fig. 3D). Staining of the tissue with antibodies to KRAS clearly stained both the nuclei and the cytoplasm of the stem cells. In addition, the cytoplasm of some other cells was also stained (Fig. 4A).

Figure 4. Staining of tumor sections with either antibodies to KRAS or to epiregulin. (A) Cluster of stem cells of poorly differentiated tumor (stage II) stained with antibodies to KRAS. The staining is essentially in the cytoplasm and the nuclei of the stem cells (double arrows). Some of the nuclei are enlarged (single arrows). There is also occasionally weak labeling in the cytoplasm of other cells. Original magnification, x400. (B) Stained section through the villi of the normal colon with antibodies to epiregulin. Staining of the cytoplasm of the villi cells is evident (double arrows), staining of a distinct population of the cells in between the villi is also evident (single arrows). Original magnification, x400. (C) Highly differentiated carcinoma cells stained with antibodies to epiregulin. The cytoplasm of the cells is weakly stained (double arrows). Original magnification, x400. (D) Moderately differentiated colon cancer cells. The cytoplasm of the cells is stained more intensively than the cytoplasm of the highly differentiated colon tumors (double arrows). Original magnification, x400.
Using antibodies to epiregulin and indirect immunocytochemistry, cytoplasmic staining of a distinct population of crypt cells of normal colon tissue was evident. Clear staining was also evident in most of the villi cells leaving the nuclei unstained (Fig. 4B). In highly differentiated tumors, weak staining with antibodies to epiregulin was evident in all carcinoma cells leaving the nuclei unstained (Fig. 4C). In moderately differentiated tumors staining of the cytoplasm was more intensive (Fig. 4D). In poorly differentiated tumors of stage I-IV, clear staining of both the cytoplasm and the nuclei of clustered stem cells was evident (Fig. 5A). In metastatic cells which had invaded through the fat tissue, a distinct population of cells was intensively stained (Fig. 5B).

**Discussion**

In the present study, we demonstrate for the first time the location and quantification of stem cells in colon cancer, using antibodies to LGR5 and immunocytochemistry. Moreover, we show a significant increase in the number of clustered stem cells at higher stages of the disease. We also show for the first time that KRAS, γ-synuclein and p53, in addition to LGR5, can serve as reliable markers of colon CSCs. Surprisingly, we could not find labeled cells with LGR5 antibodies in the normal colon tissue, apart from a few scattered cells in between the colon villi that may serve as progenitor cells to replace the damaged cells of the colon epithelium. High levels of KRAS and p53 are accepted to be resident only in the cancerous tissue (39,40). Using the above markers, our results strongly suggest that cancer tissue stem cells are different from the stem cells of normal tissue.

Of note, antibodies to epiregulin almost exclusively stained the stem cells among the non-differentiated tumor tissue. However, they also strongly stained the cytoplasm of cells in the crypt-villus axis, suggesting that these cells may represent stem cells in the normal colon wall.

In the present study, following staining with LGR5 antibodies, we found a significantly higher number of stem cells in poorly differentiated tumors than in highly differentiated tumors. This may explain the fact that patients with highly differentiated colon cancer have a lower mortality rate than patients with poorly differentiated tumors (41). This may well be due to a reflection in the number of stem cells that contribute to the proliferation, invasion and metastasis of the cancer cells.

In normal tissue homeostasis, it is clear that stem cells are involved in a careful balance between normal cell loss and renewal. Stem and progenitor cells help to maintain this precise and fine balance through their ability of self-renewal in a tightly regulated manner. However, the labeling with antibodies to LGR5, γ-synuclein and KRAS showed that normal tissue stem cells are different from those of cancerous tissue. In this regard, the gastrointestinal epithelium is unique in that cell proliferation, differentiation and apoptosis occur in an orderly fashion along the crypt-villus axis. The colonic crypt is primarily a proliferative compartment, is monoclonal and is maintained operated by stem cells. The concept of tissue stem cells capable of giving rise to all differentiated cells within a given tissue has led to the concept of a cellular hierarchy in tissues and in tumors including CRC. Thus, only a few cells may be necessary and sufficient for tissue repair or tumor regeneration. However, such a proposition also raises questions regarding the precise methods and markers to identify such populations and to define the circumstantial evidence of the origin and establishment of the early mutant stem-cell population. Thus, it is imperative that we understand what CSCs are, and their potential association with cancer in a tissue specific manner (42).

Finally, our study on the localization and quantification of colon CSCs may shed some light on therapeutic interventions for colon cancer. Since LGR5, p53, γ-synuclein and KRAS specifically labeled the stem cells of colon cancer and did not label the stem cells of the normal cells apart from a few...
scattered cells in between the colon villi that were faintly stained with antibodies to LGR5, more prominent specific markers for the stem cells in normal tissue should be explored. The intensive formation of γ-synuclein by the colon CSCs may explain their high potential to survive, to proliferate and to metastasize.

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