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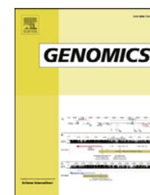

Evidence for field effect cancerization in colorectal cancer

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ABSTRACT

We compared transcript expression, and chromosomal changes on a series of tumors and surrounding tissues to determine if there is evidence of field cancerization in colorectal cancer. Epithelial cells were isolated from tumors and areas adjacent to the tumors ranging from 1 to 10 cm. Tumor abnormalities mirrored those previously reported for colon cancer and while the number and size of the chromosomal abnormalities were greatly reduced in cells from surrounding regions, many chromosome abnormalities were discernable. Interestingly, these abnormalities were not consistent across the field in the same patient samples suggesting a field of chromosomal instability surrounding the tumor. A mutator phenotype has been proposed to account for this instability which states that the genotypes of cells within a tumor would not be identical, but would share at least a single mutation in any number of genes, or a selection of genes affecting a specific pathway which provide a proliferative advantage.

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The following study was conducted to compare gene expression, copy number and loss of heterozygosity (LOH) on a series of tumor and sites distal to the tumor to determine if there is evidence of field effect cancerization. We found chromosomal abnormalities in the isolated tumor cells that had been previously reported in colorectal cancer. Epithelial cells were isolated from regions surrounding the tumor ranging from 1 to 10 cm for each of 12 patients. The number and size of the chromosomal abnormalities were greatly reduced in these cells, however many copy number and LOH events were discernable. Interestingly, these abnormalities were not consistent across the field in the same patient samples suggesting a field of chromosomal instability surrounding the tumor. A mutator phenotype has been proposed to account for this instability. This theory states that the genotypes of most cells within a tumor would not be identical, but would share at least a single mutation in any number of genes. Or this could be a collection of genes affecting a specific pathway which provide a proliferative advantage. In this scenario, the tumor would develop as a heterogeneous collection of cells all sharing a common feature of chromosomal instability. Another theory suggests that the mutator phenotype results in genetically altered cells which then clonally expand to produce tumorigenesis, but the resultant tumor carries many different clones of these original cells. Our findings show that copy number events strongly reflected widespread chromosome instability that were not consistent across sites distal to the tumor ranging from 1 to 10 cm supporting one of the mutator phenotype models for field cancerization and tumorigenesis in colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths globally with an accompanying low 5-year survival rate (~60%). Epidemiological data show that 142,950 people in the United States were diagnosed with colorectal cancer in 2007, including 73,183 men and 69,767 women. [1]. CRC can be cured if detected at an early stage. However, the early-stage disease is mostly asymptomatic; hence approximately two-thirds of patients with CRC are diagnosed at a more advanced stage. One emerging modality of cancer risk stratification is via identification of “field carcinogenesis”. The term was first used in 1953 in the landmark paper by Slaughter et al. They describe an area or "field" of epithelium that has been preconditioned by largely unknown processes so as to predispose it towards development of cancer. Since then, the terms "field cancerization" and "field defect" have been used to describe pre-malignant tissue in which new cancers are more likely to arise. Since then the concept of field cancerization in clinical oncology has received increasing attention [3]. This interest is further motivated by the exceedingly high incidence of second primary colorectal cancers occurring in approximately 300 to 400/100,000 patients between age 30–39 and 70 or over [4]. The predilection to develop neoplastic transformation should be identifiable throughout the diseased organ because the genetic and environmental surroundings that result in a neoplastic event should manifest throughout the local tissue milieu. This field cancerization concept is well established in a variety of malignancies such as the diffuse aero-digestive injury associated with smoking-induced lung cancer [5]. The clinical manifestation is the increased incidence of tobacco-related primaries in the field of injury (e.g., lung, esophagus, head and neck). Genetic mutations, such as those in the TP53 gene for example, can be found throughout the

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bronchial epithelium of cancer patients [5]. In the colon, this field of cancerization hypothesis is the rationale for colonoscopic post-polypectomy surveillance. Aside from the adenomatous polyp, there have been a number of putative biomarkers that occur earlier in the pre-dysplastic mucosa. These include gains, amplifications, losses, deletions and translocations which are the hallmarks of chromosomal instability observed in most tumor types. Copy number alterations (CNAs) typically seen in colorectal tumors may occur in low-grade dysplastic adenomas and are therefore proposed as major factors in tumorigenesis [6].

The exact mechanism of field cancerization has not been well-formulated; however theories of tumorigenesis would likely be relevant to address the question. One such theory was formulated for colon cancer in the groundbreaking study of Fearon and Voglestien in 1990 [7] wherein they hypothesized a sequential order of mutations in cancer associated genes. In this scenario, each successive mutation would confer an increased proliferative advantage. Although it was shown in adenocarcinomas of the colon, a timeline for mutations in oncogenes correlated with carcinogenesis, this model postulates that high-grade tumors would have accumulated mutations in each of the cancer-associated genes. However, subsequently it has been shown that fewer than 7% of colon cancers actually contain mutations in the three most frequently mutated genes associated with that tumor type [8].

A second scenario was suggested by Nowell in 1976 [9] whereby the observed incidence rates of cancer may be explained by mutations occurring at the normal rate in conjunction with multiple successive rounds of lineage expansion and selection. In this concept of "clonal evolution," most of these genetic variants that arise in a tumor cell population do not survive. However, those few mutants that have an additional selective growth advantage expand to become predominant subpopulations within the neoplasm. Furthermore, these selective mutants demonstrate the characteristics of more aggressive growth and increased "malignancy" that we recognize as tumor progression. The continued presence of multiple subpopulations in the neoplasm provides the basis for the heterogeneity that is also typically observed in malignant tumors.

Along similar lines the mutator phenotype model was also postulated in the 1970s by Loeb et al [10]. This model asserts that cancer is driven more efficiently via multiple pathways. Therefore the genotypes of most cells within a tumor would not be identical, but would share at least a single mutation in any number of genes which proffer a proliferative advantage. The tumor itself would develop as a heterogeneous collection of cells all sharing a common feature of chromosomal instability and having different but overlapping patterns of mutated genes. This theory is largely supported by recent Next-Gen sequencing evidence which suggests that each tumor is unique and contains hundreds to thousands of individual mutations.

In this study, we have looked at the gene expression of an enriched population of epithelial cells derived from colon tumors and adjacent tissues at variant distances from the tumor. We then performed concurrent copy number and LOH analysis on the same tumor/normal samples to identify regions which would support the concept of field cancerization and the genes that map to these regions and show up or down regulation in all of the samples studied. Using this analysis we determined that LOH events are more consistent than CNAs across the colon field. We also found a large amount of copy number variation in regions adjacent to the tumors, which suggest the presence of high levels of cellular heterogeneity.

2. Materials and methods

Specimen procurement was approved through the Health Institutional Review Board at the State University of New York at Buffalo. Specimens were obtained after surgical removal, and cells obtained from non-diagnostic, excess areas of tissue. Informed consent was obtained from study participants. The samples were de-identified and the researchers had no contact with the human subjects. Overall, 14 tumors and adjacent tissues were chosen for analysis, however, not all patient samples yielded enough quality RNA at all sites distal to the original tumors. Most of the tumors had a matching sample from 1 cm, 5 cm and 10 cm distal to the tumor. Transcript profiling was carried out on 11 samples, and of these, 7 had mRNA of adequate quality from the tumor itself and 3 sites distal to the tumor, 3 others were missing samples at 10 cm distal to the tumor and 1 had mRNA from only tumor at 1 cm. Copy number analysis was carried out 12 specimens and each had sufficient DNA from tumor and 3 sites distal to the tumor. Nine specimens used in the transcript expression studies overlapped with those used in the DNA copy number studies. This information on patient inclusion is shown in Table 1. The specimens used in the transcript expression studies for the most part, overlapped with those used in the DNA copy number studies, however the overlap was not 100%. Also, not all patient samples yielded enough quality RNA at all sites distal to the original tumors. This information on patient inclusion is shown in Table 1.

Isolation of tumor and epithelial cells: Briefly, the procurement protocol involved receipt of the extirpated specimen in the operating suite, rapid transport to the pathology department, opening and gross inspection of the specimen and removal of debris with normal saline washes at 37 °C, followed by exfoliation of cells with the edge of a glass slide [11]. The exfoliated cells were then placed into a microcentrifuge tube containing PBS with 10 mM dithiothreitol (DTT), a mucolytic agent at 37 °C. During the development of this procurement protocol, elimination of mucus was found to be necessary to prevent contamination by symbiotic bacteria present in the human intestinal tract and other cells that may have become trapped in the mucus during the exfoliation procedure. The groups of cells that were exfoliated were further dispersed into single and small groups of cells using a chelating agent (Cellstripper™, Mediatech, Herndon, VA). These washes were then followed by further enrichment with a red blood cell lysis agent (RBC Lysis Buffer, eBioscience). Final enrichment was achieved using magnetic beads coated with the Ber-Ep4 antibody, which recognizes an epitope previously documented to be expressed in colonic epithelial cells, which is considered to be specific for this cell type [12]. The resultant samples consisted of an enriched population of epithelial cells without the associated tissue contaminating normal stromal and inflammatory cells. The enriched cells were snap frozen in liquid nitrogen and stored at −80 °C. Total RNA and DNA from each sample were extracted within one month of procurement. We typically isolated ~300–500 ng of each suggesting that each epithelial isolation resulted in ~250,000 cells.

Table 1
Clinical and experimental details.

Designation in paper	Date of surgery	Tumor grade	Tumor stage	Samples	Arrays
A	40808	MD	pT3aN0pMX	T, 1, 5, 10	U133, 250 K
B	81210	MD	pT3pN2pMX	T, 1, 5, 10	250 K
C	81610	MD	pT4apN2pMX	T, 1, 5, 10	U133, 250 K
D	81710	MD	pT3pN2pMX	T, 1, 5, 10	U133, 250 K
E	40908	PD	pT3c/dpN2pMX	T, 1, 5, 10	U133, 250 K
F	41008	N/A	N/A	T, 1, 5, 10	U133, 250 K
G	82610	MD	pT3a/bpN0pMX	T, 1, 5, 10	U133, 250 K
H	41108	MD	pT3c/dpN0pMX	T, 1, 5, 10	U133 250 K
J	50508	MD	pT2pN0pMX	T, 1, 5	U133, 250 K
K	90310	MD	pT4apN0pMX	T, 1, 5, 10	250 K
L	32708	MD	pT3	T, 1, 5,	U133,
N	92010	MD	pT2a/bpN1pMX	T, 1, 5, 10	250 K
P	92310	MD	pT4apN0pMX	T, 1	U133, 250 K
S	22708	PD	pT3a/bpN0pMX	T, 1, 5,	U133

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