Human Pathology (2015) **46**, 507–513

Human
PATHOLOGY

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Original contribution

Novel molecular insights from routine genotyping of colorectal carcinomas☆,☆☆

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Received 25 October 2014; revised 24 December 2014; accepted 2 January 2015

Keywords:
Colon;
Adenocarcinoma;
Genotyping;
GNAS;
Mutations

Summary Routine tumor genotyping enables identification of concurrent mutations in tumors and reveals low-frequency mutations that may be associated with a particular tumor phenotype. We genotyped 311 colorectal carcinomas (CRCs) for 471 mutation hot spots in 41 cancer-associated genes. At least 1 mutation was present in 239 (77%) of 311 tumors. Two concurrent mutations were identified in 89 (29%) tumors, 3 mutations in 24 (8%), 4 mutations in 6 (2%), and 5 mutations in 1 tumor. *KRAS* mutations were most frequent and identified in 132 (42%) tumors, followed by *APC* in 79 (25%) and *TP53* in 64 (21%) tumors. Mutations in *PIK3CA*, *BRAF*, *CTNNB1*, and *NRAS* were identified in 41, 27, 11, and 9 cases, respectively. Rare mutations not typically associated with CRC included *AKT1* (4), *AKT2* (1), *IDH1* (1), *KIT* (1), *MAP2K1* (1), *PTEN* (2), and *GNAS* (6). *GNAS* mutations in CRC correlated with a mucinous phenotype and were present in 20% of all mucinous adenocarcinomas evaluated in this study. Among CRCs with a *PIK3CA* mutation, 77% showed concurrent mutations in other cancer-associated genes, and 4% of CRC did not neatly fit into either the chromosomal instability pathway or CpG island methylator phenotype/microsatellite instability pathway, suggesting overlapping mutational profile in some tumors. Our findings indicate that routine tumor genotyping is helpful in identifying low-frequency mutations, such as *GNAS*, that may correlate with a specific morphological phenotype and also reveal multiplicity of concurrent mutations in a significant proportion of CRC that may have significant implications for clinical trial design and personalized therapy.
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1. Introduction

Sporadic colorectal cancer (CRC) arises through 3 distinct molecular pathways. The chromosomal instability pathway (CIN) is characterized by aneuploid tumors with chromosomal gains and losses and mutations in *APC*, *KRAS*, and *TP53* [1]. Conventional adenomas are precursors of CRC that develop through the CIN pathway. The CpG island methylator phenotype (CIMP) pathway shows global hypermethylation of CpG islands throughout the genome and leads to loss of gene function through promoter hypermethylation. The microsatellite instability (MSI) pathway is related to the CIMP pathway but characterized by promoter hypermethylation of the *MLH1* gene, which leads to dysfunctional DNA mismatch repair (MMR) and increased errors during DNA replication, especially at the

☆ Statement of author contribution: M.D.S. – project design, data analysis, and manuscript preparation; E.R. – data analysis and manuscript editing; R.O. – project design and manuscript editing; N.L. – project design and manuscript editing; A.S. – project design, manuscript preparation, and editing.
☆☆ Disclosures: The authors have no conflicts of interest to disclose.

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http://dx.doi.org/10.1016/j.humpath.2015.01.005
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error-prone microsatellite repeat regions. In contrast to the CIN pathway, CRC arising through the CIMP and MSI pathways shows a predilection for the right colon, harbors *BRAF* mutations, and arises from serrated polyps [2]. The CIMP and MSI pathways are therefore also referred to as the serrated pathway of carcinogenesis.

Much of the initial work describing pathways of CRC carcinogenesis was performed using single-gene mutation analysis [2-4], and these studies were limited to detecting alterations in a few commonly mutated genes. The single-gene mutation analysis approach is also used in current clinical genetic testing for CRC and suffers from the same drawbacks. Recently, the Cancer Genome Atlas Network published a comprehensive molecular profile of CRCs [5] that confirmed the pathway-specific genetic profile described above but also identified several unique somatic mutations, such as *ARID1A*, *SOX9*, and *FAM123B*, not previously known to be associated with CRC. The study did not focus on multiplicity of mutations found within individual tumors or correlations between mutational and clinicopathological profiles of CRC. The presence of multiple concurrent mutations in CRC is likely to become therapeutically important as targeted therapies are developed against specific mutant gene targets.

Until recently, comprehensive molecular characterization of tumors was difficult to perform in clinical molecular laboratories that were primarily set up to do single-gene mutation assays. The single-gene test approach is not practical for testing a wide variety of tumors for low-frequency mutations because the number of tests that would have to be performed would be too time-consuming and poses a large burden on laboratory resources. With the advent of multiplex genotyping technologies and the rapidly dropping costs of next-generation sequencing (NGS), a comprehensive molecular characterization of tumors is becoming increasingly feasible. Understanding the genotypic complexity of specific tumor types is becoming increasingly relevant for clinical trials based on targeted drug interventions and for explaining potential mechanisms of tumor resistance to therapy. OncoMap is a tumor genotyping platform that uses single-base extension chemistry (iPlex)Sequenom/Agena Bioscience (San Diego, CA) and MALDI-TOF mass spectrophotometry (Sequenom, San Diego, CA) to genotype 471 unique mutations in 41 genes [6] (Table 1; Supplementary Table). We genotyped a series of 311 CRCs using the OncoMap platform to identify concurrent mutations within tumors and to determine whether any low-frequency mutations were associated with a distinct clinical or morphological phenotype.

Table 1 Genes included in the OncoMap platform for genotyping

Panel of genes analyzed			
ABL1	*FGFR1*	*JAK2*	*PDGFRA*
AKT1	*FGFR2*	*JAK3*	*PIK3CA*
AKT2	*FGFR3*	*KIT*	*PIK3R1*
APC	*FLT3*	*KRAS*	*PTEN*
BRAF	*GNA11*	*MAP2K1*	*RB1*
CDK4	*GNAQ*	*MET*	*RET*
CDKN2A	*GNAS*	*MLH1*	*SRC*
CSF1R	*HRAS*	*MYC*	*STK11*
CTNNB1	*IDH1*	*NPM1*	*TP53*
EGFR	*IDH2*	*NRAS*	*VHL*
ERBB2			

2. Materials and methods

2.1. Study group

Patients were consented for CRC genotyping at the time of initial registration at the Dana-Farber Cancer Institute or Brigham and Women's Hospital. Surgeons or oncologists taking care of the patients with CRC were responsible for filling out the OncoMap test requisition form. Consenting patients between August 2011 and January 2013 for whom adequate tissue material was available for genotyping were included in the study (n = 311). After identification of a unique morphological phenotype in *GNAS* mutant tumors during analysis of the initial cohort of 311 CRCs, an additional 19 mucinous colonic adenocarcinomas were also analyzed to assess the prevalence of *GNAS* mutation in this specific subgroup of CRC. The study was approved by the institutional review board at Dana-Farber Cancer Institute and Brigham and Women's Hospital.

2.2. DNA isolation and genotyping

Routinely processed hematoxylin and eosin slides from consenting patients were reviewed to determine tumor adequacy and to select the area of highest tumor percentage. The area of highest tumor percentage was then macro-dissected from either 10 unstained 4-μm tissue slides using a razor blade or 5 1.0-mm tissue cores from the corresponding tumor block for DNA isolation. In some cases, fresh tumor samples were available for DNA isolation along with a frozen section slide to determine tumor percentage. All 3 methods of DNA collection described above have been validated in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory and perform equally well with the OncoMap platform [6]. Tissue samples with at least 30% tumor and that were at least 3 mm in greatest dimension were then selected for DNA isolation. Cases that did not meet the percentage and size criteria above were excluded from the study and comprised approximately 13% of the initial study group.

DNA was isolated using a Qiasymphony (Qiagen, Valencia, CA) automated DNA extractor according to the manufacturer's recommendations. DNA was quantified using SYBR-green–based double-stranded DNA detection (Picogreen/Life Technologies, Grand Island, NY). Samples

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