REVIEW ARTICLE

The arrow of carcinogenesis

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Grant SG. The arrow of carcinogenesis. J Mol Cancer. 2017;1(1):1-6.

Many models of molecular carcinogenesis involve a multi-step progression through an accumulation of genetic and epigenetic events conferring various aspects of the transformed phenotype. Identification of tissue type-specific pathways involving distinctive genes has had some success, but it is becoming clear that such pathways may well be unique to each tumor. As an alternative, some researchers have targeted the mechanisms responsible for the accumulation of aberrant genes or gene expression, the "arrows" of transition between steps. The frequency of such genetic and epigenetic changes depends on both the lifelong exposure profile for an individual, as well as host factors, such as their innate rate of replication error and ability to remediate induced DNA damage. The totality of these effects can be evaluated functionally, however, using a number of approaches. Broadly defined, cumulative determinations of somatic mutational burden have been shown to predict subsequent cancer development, as well as demonstrating the development of genomic instability as a common characteristic of aging, that presages cancer incidence. Well-established and validated methodologies exist that can be applied to the monitoring of wellness in patients before cancer occurs.

Key words: *DNA repair; Genomic instability; Molecular carcinogenesis; Mutational burden*

When first unveiled, the somatic model of colorectal cancer (Figure 1) was thought to be the harbinger of a new era of molecular cancer biology – with simple molecular pathways for each of the major cancer types that could be mined and exploited for prevention, intervention and treatment strategies. The pathway presented included both a well-established oncogene, *ras*, as well as the gene that exemplified tumor suppression, *p53*, which at that time was touted as the universal molecular constant in carcinogenesis (1-5). Two other theoretical genes were identified by areas of consistent "loss-of-heterozygosity" at specific stages of colorectal carcinogenesis: the mutated-in-colorectal-carcinoma (*MCC*) gene on chromosome 5 that seemed to begin the process, and the deleted-in-colorectal-carcinoma (*DCC*) gene on chromosome 18, which was associated with transformation from a class II to a class III adenoma. Significant validation for the pathway was provided by the subsequent identification, cloning and characterization of both of these genes, although there is still some controversy as to whether those initially identified genes are actual the cancer "drivers" in those regions (6). In most models (including ours), the *MCC* gene has been replaced with the closely linked *APC* gene, the genetic determinant of familial adenomatous polyposis (FAP), a hereditary syndrome with a 95% incidence of colorectal cancer by age 50 (7).

Instead of this model heralding a new era of simpler cancer molecular etiology, however, it soon became clear that colorectal cancer was a special case, with a single major molecular pathway common enough to appear to be unique. With alternate pathways (some subtype specific) and branching, most major cancer types have only nonrandom associations with specific genes, rather than established pathways. For example, there are at least three overlapping pathways for lung cancer (2) and no clear pathway or pathways have emerged for breast cancer (3,4). Indeed, there are now three acknowledged molecular pathways for the development of colorectal cancer (8,9). In retrospect, expecting cancer types to have unique molecular pathways was extremely naïve, since even a casual consideration of the original proposed pathway for colorectal cancer reveals functional alternatives that have since been observed. For example, dysregulation of *Raf* can substitute for a ras mutation, having a similar effect on the activation of the MAP kinase pathway (10,11). Also, MDM2 acts as a sink for *P53* protein, so overexpression of *MDM2* is an alternative mechanism of inactivating *p53* (12). Even a single functional pathway can therefore be traversed through alternate molecular events. Thus, although the particular genes identified in Figure 1 and other proposed pathways of carcinogenesis have been used to predict risk of cancer and facilitate early detection (13,14), optimize treatment (15) and monitor treatment response (16), they require personalization for individual tumors for optimal application. There is another element to the pathway, however, that is shared by all known and hypothesized molecular models of carcinogenesis: the arrows.

ALTERNATE MECHANISMS OF HEREDITARY CARCINOGENESIS

The arrows in Figure 1 represent the mechanisms by which carcinogenesis proceeds; how the oncogenes become activated and the tumor suppressor genes inactivated. They are usually casually defined as "mutation," although we are well aware that they include types of events that go well beyond traditional point mutation (17) (Table 1).

A second, and actually more common type of hereditary colorectal cancer is hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome (4,18). Whereas, it initially appeared that the predisposition to and acceleration of onset of colorectal cancer in the other hereditary colorectal cancer syndrome, FAP, was due to inheritance of a necessary event in the common pathway of colorectal carcinogenesis, effectively shortening the pathway, HNPCC involves a set of genes not invoked in the sporadic molecular pathway, genes involved in DNA mismatch repair (MMR). The mismatch repair genes can be classified as a subset of tumour suppressor genes known as "mutator" genes; meaning that they mediate some of the mechanisms whereby accumulation of mutations can proceed to carcinogenic transformation. Indeed, although hereditary inactivating mutations in several MMR genes can contribute to carcinogenesis in specific patients and their families, in each case they still represent an additional step in the pathway of carcinogenesis (reduction to hemizygosity or homozygosity of the inherited deficient MMR gene), actually lengthening the pathway; paradoxically, however, complete loss of mismatch repair allows for a faster traverse of the pathway, with faster accumulation of the subsequent events necessary to produce the initial transformed tumor cell. Such genomic instability essentially makes the arrows themselves shorter (Figure 2).

FURTHER MECHANISMS OF GENOMIC INSTABILITY IN COLORECTAL CANCER

Another pathway of colorectal carcinogenesis has been defined largely due to its dependence on chromosomal instability (CIN) (8). Aberrant karyotypes were one of the first characteristics of tumors to be generalized (19) and can be due to ongoing chromosomal instability (20). Recent evidence suggests that loss of the *APC* gene also affects genomic stability, by inhibiting DNA base excision repair (BER) (21). We have shown consistent downregulation of DNA nucleotide excision repair (NER) in early stage breast cancer (22). With all of this evidence, it is difficult to understand how genomic instability was originally overlooked as a "hallmark" of cancer (23), and added later only as an "enabling factor" (24). This is in contrast to the "mutator" theory of carcinogenesis, which states that the acquisition of events necessary for carcinogenic transformation in a single cell could not occur within a human lifetime without the increase in frequency afforded by some type of genomic instability (25). Perhaps the resolution of these ideas would be that genomic

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Received: October 30, 2017, Accepted: December 03, 2017, Published: December 15, 2017

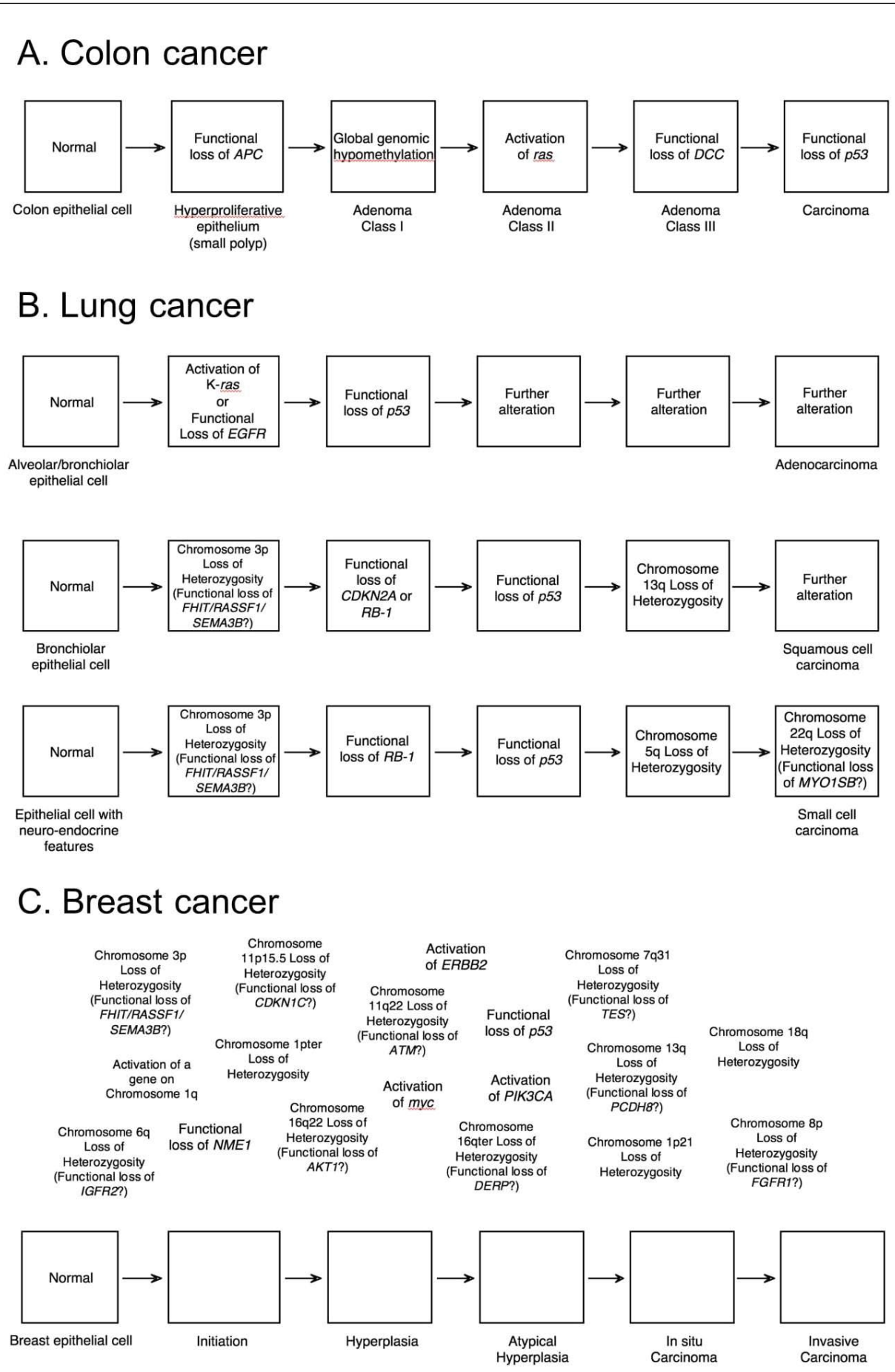

Figure 1) *Accumulation of genetic dysregulation leading to tumorigenic transformation in* **(A)** *colorectal epithelium (adapted from (1))* **(B)** *various types of lung epithelium (adapted from (2)), and* **(C)** *breast epithelium (adapted and updated from (3,4))*

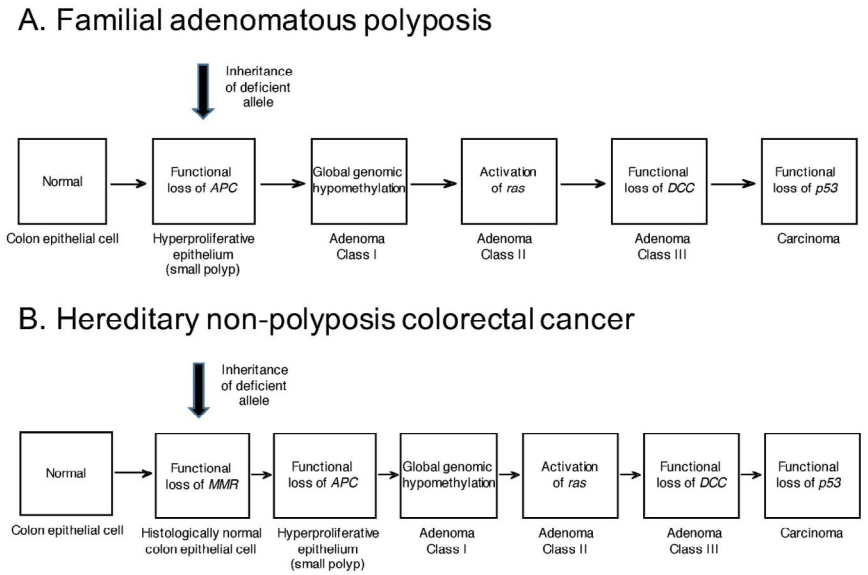

Figure 2) *Two types of hereditary predisposition for colorectal cancer. A) Inheritance of a deficient allele in a tumor suppressor gene that is part of the somatic pathway of carcinogenesis; and B) inheritance of a deficient allele in a mutator gene that results in accelerated accumulation of the events in the somatic pathway of carcinogenesis*

TABLE 1

Molecular mechanisms of carcinogenesis

Mechanisms of activation of cellular proto-oncogenes	
Mutation	Structural mutation[1] to hyperactivity Regulatory mutation[1] to overexpression Regulatory mutation[2] to unregulated expression
Epigenetic activation[3]	
Gene amplification	
Translocation/inversion/insertion	Functional juxtaposition of proto-oncogene coding regions into heterologous regulatory region
Mechanisms of inactivation of recessive tumour suppressor genes	
Mutation	Structural mutation[2] to inactivity Regulatory mutation[2] to non-expression Mutation[2] affecting mRNA processing or stability
Epigenetic inactivation[4] Gene deletion	
Translocation/inversion/insertion	Disruption of integrity of gene
Mechanisms of loss-of-heterozygosity of tumour suppressor gene	
Mutation	Structural mutation[2] to inactivity Regulatory mutation[2] to non-expression Mutation[2] affecting mRNA processing or stability
Epigenetic inactivation[4]	
Gene deletion	
Translocation/inversion/insertion	Disruption of integrity of gene
Chromosome loss Chromosome loss and duplication Mitotic recombination Gene conversion	

[1]Point mutation
[2]Point mutation, small deletion or insertion
[3]DNA hypomethylation, conformational change to euchromatin, histone deacetylation/methylation/demethylation, binding of inhibitory miRNAs
[4]DNA hypermethylation, conformational change to heterochromatin, histone acetylation/methylation/demethylation, binding of inductive miRNAs
Revised and expanded from (17)

instability is required to become cancer, but may not be required to maintain the cancer phenotype (with discussions of the possible role of genomic instability in cancer progression set aside for now).

SHARED MECHANISMS OF CARCINOGENESIS

As we have seen, there is some utility in defining the exact genes altered during carcinogenesis, even if it must be done individually for each tumour; is there any reason to similarly study the arrows? We normally think of the genetic and epigenetic mechanisms that define carcinogenesis as occurring through both endogenous processes (26) and the inevitable and unavoidable environmental, occupational, medical, lifestyle and accidental exposures we are subjected to throughout our lifetime (27). The accumulated effect of these processes, as well as any genetic mediating factors, may be referred to as the somatic mutational "burden" and has been measured in many ways (28,29).

BLOOD-BASED EVALUATIONS OF SOMATIC MUTATIONAL BURDEN AND CANCER RISK

Most impressively, screening of baseline chromosome aberration (CA) frequency (30) or frequency of micronuclei (MN) (31) in blood cells have been shown to be predictive of subsequent cancer incidence in large prospective studies. CA analysis provided a relative risk (RR) of 1.41, with a 95% confidence interval (CI) of 1.16-1.72 (32), whereas MN had a RR of 1.53 (95%CI: 1.04-2.25) (33), both of which are statistically significant. In these studies, it is not the particular genes, but the ease of traversing a standard carcinogenic pathway, that defines cancer risk (34). A related technique, quantitative analysis of induced chromosome breakage (also called "mutagen sensitivity") (35), has been applied retrospectively to patients with a number of cancer types and has produced significant odds ratios that might suggest it would also be predictive for cancer (Table 2). These chromosomal analyses are available in most children's hospitals, because they are the laboratory diagnostic standard for the cancer-prone hereditary syndrome Fanconi anemia (41,42) and a comprehensive protocol for quantitative induction of chromosome damage with a number of agents has also been recently been published (43). Blood-based somatic mutation at the *HPRT* and *GPA* reporter genes (Table 3) has also been associated with cancer incidence in retrospective studies (44). Odds ratios based on studies using these methods are given in Table 4. Cut-off values were determined as approximately 3 standard deviations above the mean of the control population. Note that for the *GPA* assay, this is similar to the criteria applied as diagnostic for the cancer-prone diseases ataxia telangiectasia (64) and Fanconi anemia (65). These odds ratios also suggest these assays might be useful in a population screen to identify individuals at increased risk for cancer. Both of these assays have been in use experimentally for over 30 years and protocols for these procedures have also recently been published in detail (66,67). Population studies with the *GPA* somatic mutation assay have shown that the incidence of high mutation frequency "outliers," perhaps indicative of the development of genomic instability in bone marrow stem cells, rises exponentially beginning at age 45 (68). Notably, this parallels the age of incidence of many solid tumors (69). Perhaps acquisition of genomic instability is the defining step in carcinogenesis, when development of a fully transformed cell becomes inevitable? Population screening and monitoring via application of one or more of these assays of the arrow of carcinogenesis should become a regular part of the evaluation of disease risk in asymptomatic human beings, rather than wait for the development of overt cancer.

TABLE 2

Association of elevated mutagen sensitivity (induced chromosome aberrations) with cancer

Cancer type	Inducing agent	Odds Ratio (95% Confidence Interval)	References
Lung	Benzo[a]pyrene diol epoxide Bleomycin	2.15 (1.39-3.33) 2.69 (1.44-5.04)	(36) (37)
Head and neck	Benzo[a]pyrene diol epoxide	1.56 (1.27-1.91)	(38)
Breast	Bleomycin	2.8 (1.7-4.5)	(39)
Melanoma	4-nitroquinoline-1-oxide	1.78 (1.12-2.84)	(40)

TABLE 3

The *HPRT* and *GPA in vivo* somatic mutation assays

	Features	Events detected
HPRT	Well-established assay, with extensive normal database Applicable to everyone except patients with Lesch-Nyhan syndrome (hereditary deficiency of HPRT) ~20 mL of fresh blood required Expensive and labor-intensive: requires cell culture and clonogenic drug selection Mutant colonies can be genetically analyzed: generate "mutational spectra" Comparable *in vitro*, animal versions	Gene-specific mutation[1] Structural mutation to inactivity Regulatory mutation to non-expression Mutation affecting mRNA processing or stability Epigenetic inactivation Gene deletion Translocation/inversion/insertion Disruption of integrity of gene
GPA	Well-established assay, with extensive normal database <1 mL of fresh blood required Inexpensive and rapid: direct flow cytometric detection of mutants Mutant phenotype cannot be conformed at the DNA level Resolution of mutants with allele-loss and loss-and-duplication phenotypes Loss-and-duplication phenotype can be resolved only in GPA (MN) heterozygotes (~50% population)	Mutation Structural mutation to inactivity Regulatory mutation to non-expression Mutation affecting mRNA processing or stability Epigenetic inactivation Gene/chromosome deletion Translocation/inversion/insertion Disruption of integrity of gene, position effects Chromosome loss Chromosome loss and duplication Mitotic recombination Gene conversion

[1]Due to location on X chromosome, limited ability to detect extragenic mechanisms
Updated and expanded from (34)

TABLE 4

Calculation of odds ratios for cancer retrospective studies of mutation frequency based on reporter genes

***HPRT* somatic mutation assay**				
Population	N	N $Mf \leq 2 \times 10^{-5}$	N $Mf > 2 \times 10^{-5}$	Odds Ratio (95% CI)
All cancer patients[1]	92	84	8	5.12 (2.03-12.88)
Disease-free controls[2]	657	645	12	
***GPA* somatic mutation assay**				
Population	N	N $Mf \leq 3 \times 10^{-5}$	N $Mf > 3 \times 10^{-5}$	Odds Ratio (95% CI)
All cancer patients[3]	98	63	35	4.24 (2.66-6.75)
Disease-free controls[4]	802	709	93	

[1]Data from cervical, bladder, endometrial, lung, head and neck, colon and prostate cancer (45), Hodgkin's disease, Ewing sarcoma and rhabdomyosarcoma (46), breast cancer (47) and lymphoma and breast, esophageal, stomach, colorectal, lung, ovarian, testicular, choriocarcinoma, squamous cell carcinoma and bladder cancer (48)

[2]Data from controls from cancer studies (46-48) and population studies (49-56)

[3]Data from adenocarcinoma, bone metastases, brain, breast, colon, germ cell, head and neck, Hodgkin's and non-Hodgkin's lymphoma, melanoma, renal, neuroblastoma, ovarian, liver, lung, cervical and thoracic cancers (57,58), testicular cancer (59) and breast cancer (60)

[4]Data from controls from cancer studies (57,60) and population studies (61-63)

N: Number; Mf: Mutation frequency

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