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Original Paper

Older individuals appear to acquire mitotically older colorectal cancers

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Abstract

The incidence of many common cancers increases with ageing. The purpose of this study is to infer whether cancer mitotic ages (total numbers of divisions since the zygote) also increase with chronological age. Mitotic ages may be inferred by counting numbers of replication errors or neutral passenger changes. Methylation at certain CpG-rich sequences or 'tags' appears to be proportional to mitotic age, because age-related increases in tag methylation are observed in mitotic tissues such as the colon. Such passenger tag methylation was measured by bisulphite sequencing from 16 colorectal cancers from differently aged male individuals. Both normal colon and cancers exhibited significant age-related increases in tag methylation, but cancer methylation was significantly higher. Therefore, older individuals appear to have mitotically older colorectal cancers. Cell division *per se* may be an important mechanism underlying the increased incidence of colorectal cancer with ageing, because the neoplastic phase appears more commonly to start from mitotically older crypt stem cells.

Keywords: colon; cancer; stem cell; genealogy; molecular clock; methylation; ageing

Introduction

Cancer is thought to progress through clonal evolution, or the successive replacement of cell populations with increasingly more fit and larger clonal populations [1]. One fundamental uncertainty about cancer progression is when progression 'starts' [1]. The start of cancer progression can be defined as the acquisition of a gatekeeper mutation that allows visible clonal expansion [2]. However, it is also possible to start at the very beginning from the zygote (Figure 1), which adds the interval between conception and a gatekeeper mutation. This early start is well-defined and more readily incorporates the observation that chronological age is one of the greatest risk factors for most carcinomas [3]. The basis for an age-related increase in cancer incidence is uncertain, but excessive cell proliferation has been associated with greater cancer risks [4].

Genealogy can organize a relationship between chronological and mitotic age (total numbers of divisions since the zygote). A colorectal cancer genome represents a genealogy that starts from the zygote (Figure 1). Because visible tumours are rare before the age of 50 years in the colon, much of this genealogy resides within normal colon, specifically crypt stem cells, which are the long-lived lineage that can accumulate errors [5]. Normal crypt stem cells are mitotic [6] and stem cell mitotic ages appear to increase with chronological age [7,8]. The mitotic age of a cancer genome is the mitotic age of its normal stem cell plus the additional divisions needed for neoplastic progression. By this logic, relative to a younger individual, a cancer arising in an older individual may have a greater mitotic age, simply because its gatekeeper mutation [2] occurred later in life in a stem cell with a greater mitotic age (Figure 1). Alternatively, there may be no correlation between patient chronological ages and the mitotic ages of their cancers if other factors trigger or promote tumourigenesis.

Although it is impractical to measure mitotic ages by directly counting human cell divisions, it may be possible to infer mitotic ages by counting numbers of replication errors. The greater the number of divisions, the greater the average number of replication errors (a molecular clock hypothesis). Recent cancer genome studies have demonstrated that somatic mutations are relatively rare even in cancers, with average frequencies <1 mutation/100 000 bases [9–11]. The rarity of somatic mutations hampers a quantitative analysis of how and when mutations accumulate with age, especially because very little is known about mutation frequencies in normal colon. Given that

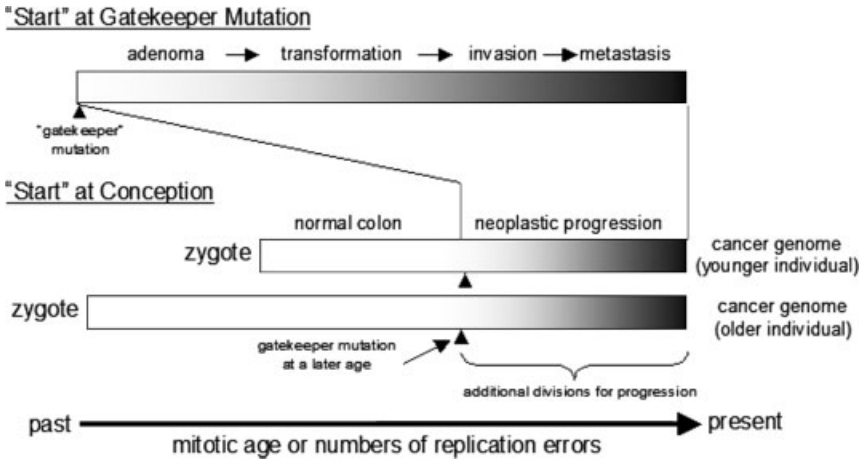

Figure 1. When does cancer start? If the start is defined by a gatekeeper mutation (black triangle), progression to cancer follows an adenoma–cancer sequence (2). However, cancer progression can also be described by a genealogy that starts from the zygote and ends at the present-day cancer genome. A cancer 'genome' may have many phenotypes during a lifetime, with a crypt stem cell phenotype early in life. The length of a genealogy represents its mitotic age, which may be inferred by counting the numbers of random replication error or passenger changes that accumulate throughout life. Older individuals may have mitotically older cancers if the 'start' of the neoplastic phase occurs in mitotically older stem cells

cancer somatic mutation frequencies are consistent with normal mutation and cell division rates [9], it is unclear how many more somatic mutations are present in a cancer genome relative to its adjacent normal mucosa. Consistent with random replication errors, the majority (>80%) of cancer somatic mutations have been classified as neutral passenger changes that appear to lack selective value [10,11].

In this study, we used somatic changes in cytosine DNA methylation to infer mitotic ages. Like sequences, DNA methylation patterns are usually copied during genome replication [12], but DNA methylation measurably increases at certain CpG islands with chronological ageing in normal mitotic tissues, such as the human colon [13]. Therefore, it is possible to infer relative proportions of somatic changes before and after a gatekeeper mutation by comparing relative numbers of methylation changes in normal versus neoplastic colon. DNA methylation measured by bisulphite sequencing in certain CpG-rich sequences or 'tags' appears to represent random replication errors or neutral passenger changes, because average methylation increases with chronological age in normal colon and patterns differ within and between crypts [7,8]. Here we measure these tags in cancer cells from differently aged individuals and infer that mitotically older cancers arise in older individuals.

Materials and methods

Normal colon ($n = 28$) or cancer tissues ($n = 16$) were obtained from differently aged patients at the Norris Cancer Center. The study was approved by our Institution Review Board. Some of the normal colon data have been reported previously [7]. Individual normal colon crypts (~2000 cells) or cancer gland fragments (~2000–10 000 cells) were isolated using an EDTA solution [7]. DNA from each crypt or cancer fragment (>90% epithelial cells) was bisulphite-treated and then amplified by PCR with primers specific to CpG-rich regions or 'tags' of CSX and BGN (Figure 2A). Approximately eight cloned PCR products were sequenced from each gland or fragment. Between five and nine normal crypts were analysed from each colon. BGN tags were sampled from ~14 fragments per cancer and CSX tags were sampled from ~two fragments per cancer (Table 1). To help validate that tag methylation levels reflect cancer mitotic ages, glands were sampled from different parts of the same cancer, at least 2 cm apart. Tag methylation levels should correlate between different parts of the same cancer, because 'left' and 'right' portions of the same cancer should have similar mitotic ages.

Because methylation patterns were variable between tags from a given tissue, average values were used for analysis. Average percentage methylation was calculated from the proportions of methylated versus non-methylated CpG sites (eight CpGs per CSX tag, eight or nine CpGs per BGN tag) for all alleles from each tissue. Statistical analysis used a t-test for comparisons between tumour and normal tags, and linear regression to test for age-related methylation.

Results

Methylation was measured in two CpG-rich tags (BGN and CSX; Figure 2A) from normal colon crypts and small 2000–10 000 cell gland fragments from 16 cancers by bisulphite sequencing of cloned PCR products (Table 1). The CSX tag is in the 3′ untranslated region of its gene and the BGN tag is present in the body of the first exon of its gene. Both the BGN and CSX tags exhibit age-related increases in methylation in normal human colon and T cells [7,14]. The CSX

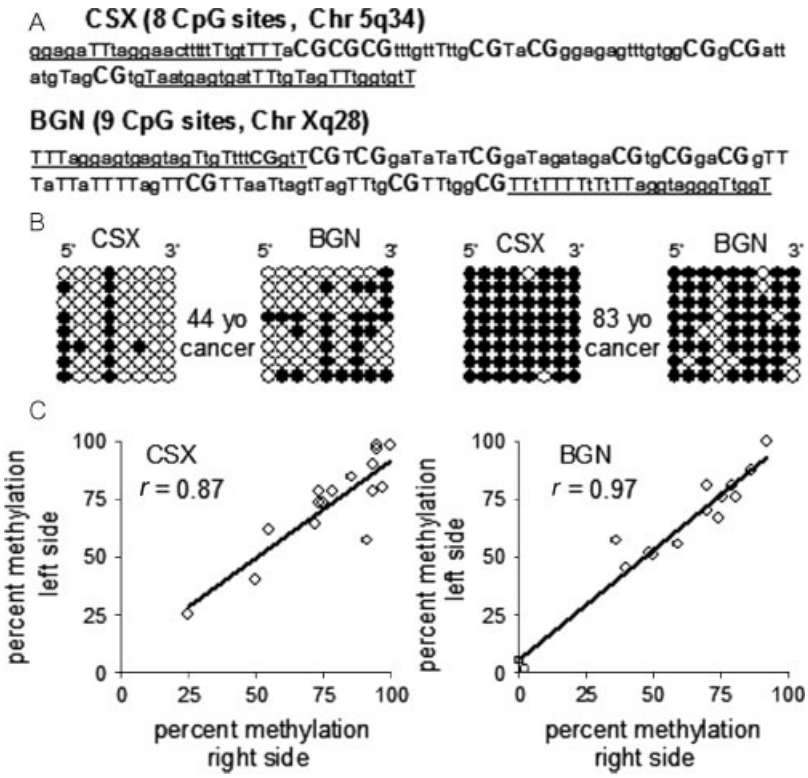

Figure 2. BGN and CSX passenger methylation. (A) Sequences of the CSX and BGN tags after bisulphite treatment. Primer sequences are underlined. (B) Illustrated are eight tags (filled circles are methlylated CpG sites arranged horizontally in a 5′ → 3′ order) sampled from cancer gland fragments from two patients of different ages. Consistent with random replication errors, tag patterns are heterogeneous within cancer glands. (C) If tag methylation arises from random replication errors, tag patterns should be heterogeneous within a cancer, yet average methylation levels should also be similar between different parts of the same cancer because different parts of the same clonal expansion should have similar mitotic ages. Consistent with this expectation, average tag methylation levels correlated between opposite sides ('left' versus 'right') of the same cancer

Table 1. Colorectal cancers

Cancer	Age at cancer	Size/ stage	Average CSX methylation (no. of glands examined)*	Average BGN methylation (no. of glands examined)
1	37	5.0/C	68.1 (2)	2.8 (2)
2	43	5.5/C	73.4 (2)	42.6 (14)
3	44	9.0/C	25.0 (2)	46.7 (14)
4	46	4.5/B	74.2 (2)	50.2 (14)
5	50	4.5/A	74.2 (2)	77.9 (14)
6	51	5.5/C	45.3 (2)	2.1 (14)
7	53	4.5/C	85.9 (2)	75.4 (14)
8	61	7.5/C	85.2 (2)	57.2 (14)
9	62	7.0/B	58.6 (2)	86.9 (9)
10	65	3.0/A	92.2 (2)	87.1 (13)
11	71	5.5/C	99.2 (2)	96.2 (2)
12	75	5.5/B	75.8 (2)	50.4 (2)
13	79	4.0/A	96.9 (2)	70.0 (14)
14	83	8.0/B	88.3 (2)	79.7 (14)
15	85	7.5/D	96.1 (2)	75.7 (14)
16	98	6.0/B	78.1 (2)	70.5 (14)

* Glands were sampled from two different parts ('left' or 'right') of the same cancer.

tag also exhibits age-related increases in methylation in normal human small intestines and endometrium (15,16). Tag methylation changes are considered 'passenger' (selectively neutral) changes because their genes are not expressed in the colon [7]. BGN is on the X chromosome and its tag was only measured from male individuals. All of the cancers were from males. Average methylation values are hypothesized to be proportional to lifetime numbers of divisions or mitotic age [8].

Cancer tag methylation patterns were heterogeneous (Figure 2A). Such cancer pattern tag heterogeneity could arise because replication errors should continue to accumulate independently in different cancer cells during the divisions after transformation. To test this scenario, tag methylation levels were compared between fragments from different parts ('left' versus 'right') of the same cancer (Figure 2B). Consistent with a measure of mitotic age (a property that should be similar between different parts of the same cancer), average tag methylation correlated between right and left sides of the same cancer ($r = 0.87$ for CSX, $r = 0.97$ for BGN).

Average cancer tag methylation was significantly greater ($p < 0.001$) than in normal crypts (Figure 3). Both normal crypts and cancers demonstrated significant age-related increases in average BGN or CSX tag methylation (Figure 4). The cancer trend lines exhibited slightly greater increases with ageing relative to normal crypts, but these differences were not significant. Cancer tag methylation was generally about two to three times greater than normal colon from similarly aged individuals.

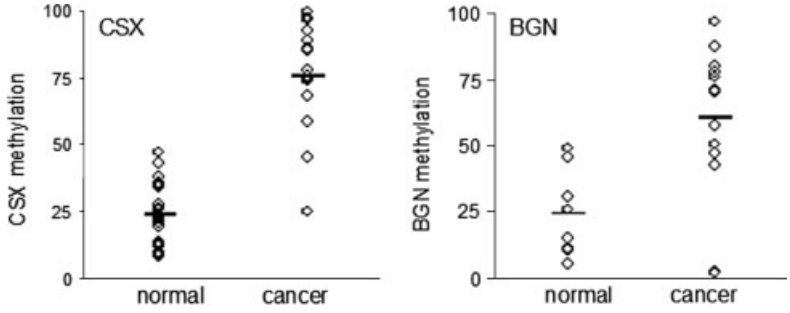

Figure 3. Average CSX and BGN tag methylation was significantly (both $p < 0.001$) greater in cancers compared to normal crypts

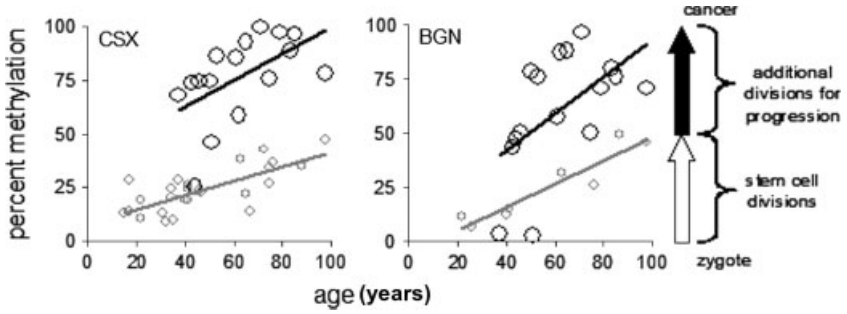

Figure 4. Significant age-related increases were observed for average CSX ($p < 0.0001$) or BGN ($p < 0.001$) tag methylation in normal crypts (grey circles and lines). Significant age-related increases in average CSX ($p = 0.030$) or BGN ($p = 0.028$) tag methylation were also observed for the cancers (black circles and lines). The age-related slopes were not significantly different between normal crypts and cancer (interaction p values of 0.16 for CSX, 0.43 for BGN). It appears that neoplastic phase intervals are similar regardless of the chronological age at cancer, and older individuals have mitotically older cancers because the neoplastic phase may start later in a mitotically older stem cell

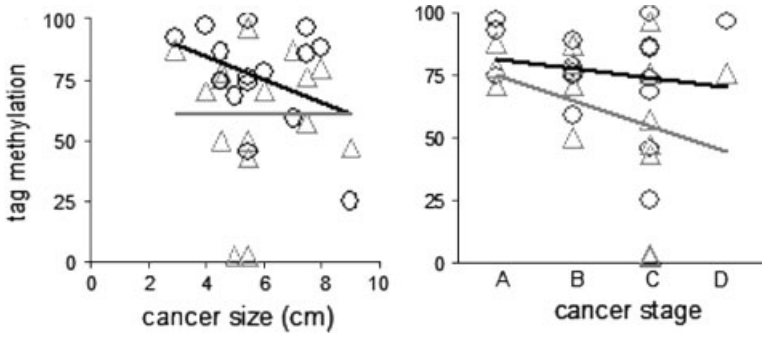

Figure 5. There were no significant relationships ($p > 0.10$) between average cancer tag methylation and cancer size or stage. CSX tag values are black circles and lines, BGN tag values are grey triangles and lines

To test whether tag methylation correlated better with visible measures of progression, cancer tag methylation was plotted with respect to cancer stage and size (Figure 5). There were negative but non-significant correlations ($p > 0.10$) between cancer tag methylation and cancer size or stage. In summary, passenger tag methylation significantly increased with chronological ageing in normal colon and cancers, but tag methylation levels did not correlate with visible measures of tumour progression (size and stage).

Discussion

Progression to cancer is thought to require alterations in a small number of cellular properties [17]. When and where these alterations occur is uncertain, but any model that can account for the exponential increase in cancer incidence with ageing must include the time between birth and cancer discovery. Indeed, the increase in cancer incidence with ageing can be explained by a small number (<10) of rate-limiting steps that start to accumulate from birth [3]. This process can be represented by a genealogy that starts from the zygote, passes through normal colon and ends with a present-day cancer genome (Figure 1).

The current data are consistent with the idea that older individuals have mitotically older cancers, because passenger cancer tag methylation significantly increases with age. At least some of the cancer tag methylation appeared to first accumulate in

normal-appearing colon, because age-related increases in tag methylation were also observed in normal colon. A simple model is that cancer mitotic age is the mitotic age of the 'normal' stem cell at the time of a gatekeeper mutation, plus the additional divisions needed for neoplastic progression (Figure 1). Age-related increases in normal stem cell mitotic ages appear largely responsible for overall increases in cancer mitotic ages with chronological ageing, because the rates of age-related changes in cancer and normal crypt methylation were not significantly different. On average it appears that neoplastic progression to cancer after a gatekeeper mutation requires similar numbers of divisions regardless of when the gatekeeper mutation occurs.

The relative paucity of somatic mutations in cancer genomes (<1 mutation/100 000 bases) is consistent with normal mutation rates, given that mutations may accumulate both in normal colon and during the additional divisions needed for progression [9]. The relative numbers of somatic mutations that accumulate before a gatekeeper mutation are unknown because mutation frequencies in normal colon are uncertain. Age-related increases in somatic mutations in normal human colon have been documented for the *O*-acetyl transferase gene through periodic acid–Schiff (PAS) staining, with mutant crypt frequencies increasing to approximately one to two/1000 crypts in the elderly [18,19], but base mutation frequencies cannot be calculated because the mutation target of this gene is unknown. By counting mutations in adenomas and cancers, about half the somatic mutations present in a cancer were inferred to accumulate in normal colon before a gatekeeper mutation [20]. With respect to our passenger tag data, cancers appeared to have about two to three times more changes relative to normal colon from similarly aged individuals, suggesting that either cell division or methylation error rates increase relative to normal colon during neoplastic progression. Greater cancer mitotic ages relative to surrounding normal colon would be more consistent with the uncontrolled proliferation hallmark of cancer [17].

Cancers may more frequently arise in older individuals because they have greater numbers of alterations in their normal stem cells, and therefore have greater risks of randomly accumulating a proper combination of selective mutations [21]. Starting from the zygote, random replication errors in normal-appearing colon can help to explain the large proportions (>80%) of passenger mutations in cancer genomes and the marked heterogeneity of mutations between different cancer genomes [11]. Some selective 'driver' mutations may first start as passenger mutations in normal crypts (ie precede a gatekeeper mutation) because tumourigenesis may sometimes require combinations of mutations [22].

The importance of an appropriate 'start' when considering cancer mitotic age is illustrated by the comparisons between cancers of different sizes and stages (Figure 5). Tag methylation did not significantly correlate with tumour size or stage, even though additional divisions are likely required for growth, invasion or metastasis. However, these additional divisions appeared to be relatively insignificant compared to the total numbers of divisions between the zygote and transformation. Some cancers may already possess their full malignant potential at the time of transformation [23,24] and therefore relatively few additional divisions may be needed for invasion and metastases. Consistent with relatively rapid tumour progression after transformation, only a few more somatic mutations (3%) were present in a metastasis compared to its primary tumour [20].

In summary, inferring that older individuals have mitotically older cancers may help to explain why colorectal cancers become more common with ageing. With cell division, replication errors become more frequent, increasing the probability of accumulating an appropriate and perhaps unique combination of driver alterations within a single cell. Further studies are needed to determine whether other genomic regions also exhibit similar greater numbers of somatic changes with cell division and ageing. Genealogy is another way to organize genomic changes into understandable evolutionary pathways.

Acknowledgements

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