The Combination of Genetic Instability and Clonal Expansion Predicts Progression to Esophageal Adenocarcinoma

Carlo C. Maley, Patricia C. Galipeau, Xiaohong Li, Carissa A. Sanchez, Thomas G. Paulson, Patricia L. Blount, and Brian J. Reid

ABSTRACT

There is debate in the literature over the relative importance of genetic instability and clonal expansion during progression to cancer. Barrett’s esophagus is a uniquely suited model to investigate neoplastic progression prospectively because periodic endoscopic biopsy surveillance is recommended for early detection of esophageal adenocarcinoma. We hypothesized that expansion of clones with genetic instability would predict progression to esophageal adenocarcinoma. We measured p16 (CDKN2A/INK4A) lesions (loss of heterozygosity, mutations, and CpG island methylation), p53 (TP53) lesions (loss of heterozygosity, mutation) and ploidy abnormalities (aneuploidy, tetraploidy) within each Barrett’s esophagus segment of a cohort of 267 research participants, who were followed prospectively with cancer as an outcome. We defined the size of a lesion as the fraction of cells with the lesion multiplied by the length of the Barrett’s esophagus segment. A Cox proportional hazards regression indicates that the sizes of clones with p53 loss of heterozygosity (relative risk = 1.27 ᵗ for an x cm clone; 95% confidence interval, 1.07–1.50) or ploidy abnormalities (relative risk = 1.31 ᵗ for an x cm clone; 95% confidence interval, 1.07–1.60) predict progression to esophageal adenocarcinoma better than the mere presence of such clones (likelihood ratio test, P < 0.01). Controlling for length of the Barrett’s esophagus segment had little effect. The size of a clone with a p16 lesion is not a significant predictor of esophageal adenocarcinoma when we controlled for p53 loss of heterozygosity status. The combination of clonal expansion and genetic instability is a better predictor of cancer outcome than either alone. This implies that interventions that limit expansion of genetically unstable clones may reduce risk of progression to cancer.

INTRODUCTION

Multistage carcinogenesis is an evolutionary process in which genetic instability generates new clones and natural selection among such variants drives clonal expansions (1–6). The number of genetic lesions necessary to produce a cancer is unknown for most tissues but has been variously estimated to be 2 to 12 (7–9). Normal mutation rates in the absence of clonal expansion cannot account for the accumulation of so many lesions (10). There has been much debate in the literature as to the relative importance of a mutator phenotype (10, 11) and genetic instability (3, 12) versus clonal expansion (4, 5) in neoplastic progression.

Both genetic instability and clonal expansions are commonly observed in neoplastic progression, and it is likely that the two factors cooperate in clonal evolution (11, 13). If this is true, then expansion of a genetically unstable clone in a premalignant neoplasm will associate with risk of progression to cancer.

The debate over the relative importance of genetic instability and clonal expansion has evolved largely around colon cancer, for which elegant studies can assess intermediate stages of adenoma progression (14). However, abnormalities cannot be studied prospectively for progression to cancer because colonic polyps are typically removed when detected.

Barrett’s esophagus is a uniquely suited model to investigate in vivo clonal expansion and genetic instability prospectively as predictors of progression to cancer in humans (15–18). Periodic endoscopic biopsy surveillance of Barrett’s esophagus is recommended for early detection of esophageal adenocarcinoma (19, 20). Barrett’s esophagus is a neoplastic condition that is associated with a 30- to 40-fold increase in the risk of developing esophageal adenocarcinoma (21–23). Barrett’s epithelium meets the criteria for a neoplasm (24) in that it is hyperproliferative relative to normal squamous epithelium (25) and generally clonal (18), and progression to esophageal adenocarcinoma is associated with clonal evolution (2). Two of the most commonly lost tumor suppressor genes in human cancers, p53 (TP53) and p16 (CDKN2A/INK4A), are also lost in Barrett’s esophagus (2). Clonal expansions of cells with p16 lesions are observed in more than 85% of Barrett’s esophagus segments (18) and tend to fill the entire Barrett’s segment (24). There is evidence for genetic instability in the form of loss of heterozygosity (LOH) even in the earliest stages of the disease (18). LOH on chromosome 17p at the p53 locus is associated with a 16-fold increase in the risk of progression to esophageal adenocarcinoma (16). The presence of aneuploidy [relative risk (RR) = 9.5; 95% confidence interval (CI), 4.9–18] and tetraploidy (i.e., 4N fraction >6%, RR = 11.7; 95% CI, 6.2–22) are also associated with progression to esophageal adenocarcinoma (17).

On the basis of the evolutionary theory of neoplastic progression, the size of the Barrett’s segment, as a surrogate for the evolving cell population size, should associate with progression to esophageal adenocarcinoma. One retrospective, hospital-based, case-control study in Rotterdam excluded segments shorter than 3 cm and found that a doubling in length of the Barrett’s segment was associated with a 1.7 odds ratio for esophageal adenocarcinoma (26). Another retrospective, case-control study from the Hines Veterans Affairs Barrett’s Esophagus Cohort (27), including all segment lengths, reported an odds ratio of 2.48 per cm. However, an earlier prospective study of the Seattle Barrett’s Esophagus Cohort reported only a nonsignificant trend with a 5-cm difference in segment length associated with a 1.7-fold increase in cancer risk (28). The relative weakness of the evidence for an effect of segment length on cancer risk suggests the size of the relevant population of cells, i.e., those that progress to cancer, may not have been assessed. It may be that the number of cells that are genetically unstable is more important for progression than the number of proliferating cells. Therefore, we hypothesized that the size of the clone with p53 lesions, or aneuploidy, or tetraploidy would predict progression to esophageal adenocarcinoma. Because clones with p53 lesions seem to require a p16 lesion before they can expand (24), we hypothesized in addition that the size of clones with p16 lesions would also predict progression to esophageal adenocarcinoma.

Carcinogenesis is often separated into processes of initiation and promotion. The initiating event in Barrett’s esophagus is currently unknown but is associated with chronic gastro-esophageal reflux (29,
30). By measuring the sizes of clones with genetic lesions in Barrett’s esophagus and following the patients to cancer outcome, we may determine the relative importance of genetic instability and clonal expansion in promotion. Although understanding initiation will be important for cancer prevention efforts, measuring lesions during progression is likely to be clinically relevant for both prognosis and cancer prevention.

In this study, we measured the size of clones with p53 lesions, p16 lesions, aneuploidy, and tetraploidy in the neoplasms of participants with Barrett’s esophagus at baseline and followed the research participants with serial endoscopies for up to 8 years with progression or lack of progression to esophageal adenocarcinoma as end points. Below, we present the first prospective data, with cancer as an end point, that addresses the roles of genetic instability and clonal expansion in a human, sporadic, premalignant neoplasm.

MATERIALS AND METHODS

Patients. Research participants were members of the Seattle Barrett’s Esophagus Cohort with intestinalized metaplastic epithelium documented in baseline endoscopic biopsies between January 5, 1995, and November 19, 2003. Fifty participants were excluded because of a lack of any follow-up data, which left 267 eligible participants. The Seattle Barrett’s Esophagus Study was approved by the Human Subjects Division of the University of Washington in 1983 and was renewed annually thereafter with reciprocity from the institutional review board (IRB) of the Fred Hutchinson Cancer Research Center (FHCRC) from 1993 to 2001. Since 2001, the study has been approved annually by the IRB of the FHCRC with reciprocity from the Human Subjects Division of the University of Washington.

Endoscopic Biopsies. Endoscopic procedures and the collection of biopsies have been described previously (31). One biopsy every 2 cm was analyzed for molecular data in most cases. In 23 participants, one biopsy was analyzed per 1 cm. Excluding these participants from the analysis does not change the significance of the results.

Flow Cytometry. Biopsies were flow-sorted based on Ki67 status and DNA content as described previously (17, 31–33). Each biopsy was separated into diploid proliferating cells (Ki67 +) and either aneuploid cells, if they were present, or cells with 4N DNA content. Tetraploid clones were defined as 4N fractions >6% (17).

DNA Extraction and Amplification. DNA was extracted from both of the flow-purified cell populations with either standard phenol/chloroform or the Puregene DNA Isolation Kit as recommended by the manufacturer (Gentra Systems, Inc, Minneapolis, MN). Whole genome amplification with primer extension preamplification was performed as described previously (32) for each sorted fraction and three constitutive controls per participant.

Loss of Heterozygosity. LOH analysis was performed on the flow-purified fractions, as described previously (32, 34) yielding information for p53 LOH in 256 participants and for p16 LOH in 259 participants.

Methylation. Genomic DNA was evaluated for p16 promoter methylation in 317 flow-purified fractions from 121 participants with methods for bisulfite treatment and methylation-specific PCR described previously (18). Human genomic DNA, treated in vitro with Sss I methyltransferase (New England Biolabs, Beverly, MA), was used as the methylated control. In a subset of cases, promoter methylation was determined and/or verified by direct sequencing PCR products of bisulfite-treated genomic DNA, as described previously (18).

DNA Sequencing. Genomic or primer extension preamplification DNA was sequenced with either BigDye or BigDyeV3 Terminator cycle sequencing (Applied Biosystems, Foster City, CA) on an ABI 377, 3730, or 3700 DNA sequencer. Wild-type sequences for each participant were confirmed with constitutive samples. All of the mutations were confirmed by at least two independent PCR and sequencing reactions, and, in cases of ambiguity, by direct sequencing of genomic DNA. Evaluation of mutation of exons 5 to 9 of the p53 gene was performed on 839 flow-purified fractions from 236 participants with conditions described previously (35). Mutation analysis of exon 2 of the p16 gene was performed on 1,195 flow-purified fractions from 239 participants with an aliquot of genomic DNA that had undergone whole genome amplification (primer extension preamplification), as described previously (18).

Statistical Analysis. Follow-up time was calculated as the time between the baseline endoscopy and either the last endoscopy before the end of data collection (November 19, 2003) or the first endoscopy that resulted in a diagnosis of esophageal adenocarcinoma. The length of the evaluated segment was measured as the level (distance in cm from the incisors) of the most distal biopsy evaluated for molecular markers minus the level of the most proximal biopsy evaluated for molecular markers plus 1. We adjusted the calculated fraction of flow-sorted cells that carry a lesion within a Barrett’s segment by excluding nonproliferating diploid cells as described previously (24). We define the size of a lesion to be the product of the segment length and the fraction of cells in the biopsies from the participant that carry the lesion. Thus the size is an estimate of the area covered by the clone measured in cm. The size of a clone is different from the number of cm over which a clone has expanded because a clone may only comprise a portion of the cells sampled from each level.

The Cox proportional hazards regression method was used to measure the association of lesion expanses and sizes with cancer outcome. Likelihood ratio tests (LRTs) were used to assess the benefit of adding additional predictor variables to the Cox models. Backward and forward stepwise selection based on the Akaike information criterion (36) in the R statistics package was used to derive a unified multivariate Cox model starting from a model that included all of the univariate predictors found to be statistically significant predictors of cancer outcome. A logistic regression was used to measure the association of p16-deficient clone sizes with the presence of a p53 lesion.

RESULTS

The 267 participants in the cohort were followed for an average of 4.4 years (range, 0.1–8.4 years). The age and sex of participants with p53 LOH, aneuploidy, or tetraploidy were statistically indistinguishable from participants without those lesions (Table 1). The Barrett’s segment lengths of participants with p53 LOH, aneuploidy, or tetraploidy were longer than participants without those lesions (2-sided t test, P < 0.001). Neither age nor sex had a statistically significant association with cancer outcome (Cox proportional hazards: age RR = 1.01, 95% CI: 0.98–1.04; sex RR = 1.15, 95% CI: 0.50–2.67). Patients who were lost to follow-up did not have a statistically significant difference compared with the cohort of study in the frequency or clone size of p53 LOH, p16 lesions, aneuploidy/tetraploidy, or segment length (Wilcoxon rank-sum and χ² tests, P > 0.05).

When the cohort is restricted to the 53 participants that have a clone with p53 LOH, the size of the p53 LOH clone predicts cancer outcome (Table 2; Fig. 1, univariate Cox model RR = 3.3 for 5 cm of the clone, 95% CI: 1.4–7.6). The size of clones with either p53 LOH or p53 mutation also predicts cancer outcome, but the size of clones with a p53 mutation is not a significant predictor of progression to cancer when analyzed alone (RR = 1.84 for 5 cm of the clone, 95% CI: 0.56–5.98). Among participants with an aneuploid or tetraploid clone, clone size also predicts progression to cancer (RR = 3.9 for 5 cm of the clone, 95% CI: 1.4–10.5), although neither aneuploidy alone nor tetraploidy alone reached statistical significance as predictors. Although many of the predictors are significantly associated with progression to cancer, point estimates for their risks vary over large ranges, as indicated by the width of the confidence intervals. The size of a clone with a p16 lesion (LOH, mutation, or methylation) predicts cancer outcome (Table 2), but this effect disappears when participants with a p16 lesion are stratified by the presence or absence of p53 LOH (Tables 2 and 3). Participants with large clones with a p16 lesion also tend to have a p53 LOH lesion (logistic regression odds ratio = 1.16 for an x cm clone, 95% CI: 1.06–1.26).

We evaluated whether previously known predictors of cancer out-

6 Internet address: http://www.r-project.org.
come were significantly improved by the addition of clone size measurements. Adding the size of the p53 LOH clone to segment length alone significantly improved prediction of cancer outcome (Table 3, row 1; LRT P < 0.01). Accounting for the size of p53 LOH clones along with the presence of p53 LOH predicted cancer better than the presence of a p53 LOH lesion alone (Table 3, row 3; LRT P < 0.01). Similarly, accounting for the size of aneuploid/tetraploid clones along with the presence of an aneuploid/tetraploid clone predicted cancer better than the presence of an aneuploid/tetraploid clone alone (Table 3, row 4; LRT P < 0.01). Combining p53 LOH clone size and aneuploid/tetraploid clone size predicted cancer better than either clone size alone (Table 3, row 5; LRT P < 0.001). Thus, information on clone sizes significantly improves our estimates of risk for progressing to cancer in all analyses. The multivariate Cox model with the optimal Akaike information criterion included only the size and presence of p53 LOH clones and the size and presence of aneuploid/tetraploid clones (Table 3, last row).

The cohort only included 12 cases of participants with p53 LOH and no flow abnormality at baseline that developed aneuploidy or tetraploidy in follow-up. We were, thus, unable to determine whether the size of clones with a p53 lesion predicts the future development of aneuploidy or tetraploidy. p16 LOH clone size did not significantly predict progression to aneuploidy or tetraploidy (RR = 1.10 for 5 cm of the clone, 95% CI: 0.62–1.93; n = 100).

If the six participants with less than 6 months of follow-up are excluded, then the effect of p53 LOH clone size on cancer outcome was slightly weakened (previously RR = 3.30, with six participants excluded RR = 2.70 for 5 cm of the clone, 95% CI: 1.00–7.10). However, the effect of aneuploid/tetraploid clone size was slightly strengthened (previously RR = 3.86, with six participants excluded RR = 4.32 for 5 cm of the clone, 95% CI: 1.40–12.99).

In contrast to the results for the absolute sizes of clones, the fraction of the Barrett’s segment covered by a clone with a p53 LOH lesion does not predict cancer (RR = 1.08, 95% CI: 0.30–3.82). Nor does the fraction of the segment covered by an aneuploid or tetraploid clone predict cancer outcome (RR = 1.02, 95% CI: 0.25–4.23).

**DISCUSSION**

Evolution is driven by both mutation and natural selection (36). The debates over genetic instability and clonal expansion in neoplastic progression have really been debates over the relative importance of

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**Table 1** Characteristics of the cohort

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Patients with</th>
<th>Patients with</th>
<th>Full cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p53 LOH</td>
<td>aneuploidy or</td>
<td></td>
</tr>
<tr>
<td>Total subjects</td>
<td>52</td>
<td>47</td>
<td>267</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>41 (79%)</td>
<td>36 (77%)</td>
<td>211 (79%)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (21%)</td>
<td>11 (23%)</td>
<td>56 (21%)</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>1 (2%)</td>
<td>4 (9%)</td>
<td>16 (6%)</td>
</tr>
<tr>
<td>40–49</td>
<td>12 (23%)</td>
<td>9 (19%)</td>
<td>49 (18%)</td>
</tr>
<tr>
<td>50–59</td>
<td>16 (31%)</td>
<td>13 (28%)</td>
<td>68 (25%)</td>
</tr>
<tr>
<td>60–69</td>
<td>16 (31%)</td>
<td>13 (28%)</td>
<td>64 (24%)</td>
</tr>
<tr>
<td>70–79</td>
<td>5 (10%)</td>
<td>6 (13%)</td>
<td>60 (22%)</td>
</tr>
<tr>
<td>≥80</td>
<td>2 (4%)</td>
<td>2 (4%)</td>
<td>10 (4%)</td>
</tr>
<tr>
<td>Segment Length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 cm</td>
<td>6 (12%)</td>
<td>8 (17%)</td>
<td>85 (32%)</td>
</tr>
<tr>
<td>3–6 cm</td>
<td>21 (40%)</td>
<td>21 (45%)</td>
<td>106 (40%)</td>
</tr>
<tr>
<td>7–10 cm</td>
<td>14 (27%)</td>
<td>11 (23%)</td>
<td>55 (21%)</td>
</tr>
<tr>
<td>&gt;10 cm</td>
<td>11 (21%)</td>
<td>7 (15%)</td>
<td>21 (8%)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Evolution is driven by both mutation and natural selection (36). The debates over genetic instability and clonal expansion in neoplastic progression have really been debates over the relative importance of

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**Table 2** Clone size and the risk for progression to esophageal adenocarcinoma

<table>
<thead>
<tr>
<th>Clone with predictor</th>
<th>Follow-up in person-years</th>
<th>Number with cancer</th>
<th>RR for an x cm clone (95% CI)</th>
<th>RR for 5 cm of a clone (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 LOH</td>
<td>53</td>
<td>167</td>
<td>26</td>
<td>1.27 (1.07–1.50)**</td>
</tr>
<tr>
<td>p53 mutation</td>
<td>39</td>
<td>138</td>
<td>16</td>
<td>1.13 (0.89–1.43)</td>
</tr>
<tr>
<td>Either p53 lesion</td>
<td>63</td>
<td>213</td>
<td>27</td>
<td>1.26 (1.08–1.47)**</td>
</tr>
<tr>
<td>Both p53 mutation and LOH</td>
<td>31</td>
<td>104</td>
<td>14</td>
<td>1.23 (0.85–1.84)</td>
</tr>
<tr>
<td>p16 LOH</td>
<td>157</td>
<td>685</td>
<td>31</td>
<td>1.09 (0.99–1.20)</td>
</tr>
<tr>
<td>p16 mutation</td>
<td>34</td>
<td>146</td>
<td>5</td>
<td>0.82 (0.50–1.37)</td>
</tr>
<tr>
<td>p16 methylation</td>
<td>74</td>
<td>313</td>
<td>16</td>
<td>1.13 (0.99–1.29)</td>
</tr>
<tr>
<td>p16 null</td>
<td>58</td>
<td>237</td>
<td>15</td>
<td>0.89 (0.62–1.26)</td>
</tr>
<tr>
<td>Amy p16 lesion</td>
<td>194</td>
<td>869</td>
<td>31</td>
<td>1.13 (1.03–1.23)**</td>
</tr>
<tr>
<td>p16 lesion without p53 LOH</td>
<td>146</td>
<td>722</td>
<td>8</td>
<td>1.05 (0.85–1.31)</td>
</tr>
<tr>
<td>p16 lesion with p53 LOH</td>
<td>45</td>
<td>134</td>
<td>23</td>
<td>1.07 (0.93–1.21)</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>32</td>
<td>80</td>
<td>17</td>
<td>1.28 (0.97–1.69)</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>26</td>
<td>73</td>
<td>14</td>
<td>1.56 (0.62–3.94)</td>
</tr>
<tr>
<td>Aneuploid/tetraploid</td>
<td>47</td>
<td>134</td>
<td>24</td>
<td>1.31 (1.07–1.60)**</td>
</tr>
<tr>
<td>p53 LOH or aneuploid/tetraploid</td>
<td>65</td>
<td>222</td>
<td>30</td>
<td>1.33 (1.15–10.55)**</td>
</tr>
</tbody>
</table>

**NOTE:** Relative risks are an exponential in the size of the clone; therefore, we have calculated the relative risk (RR) of a clone making up 5 cm worth of the Barrett’s segment. The RR for 5 cm of a clone is included as an example to facilitate intuition for the exponential risk functions of Cox regressions (e.g., 5 cm of a p53 LOH clone RR = 1.27x = 3.30). Bold values are statistically significant.

**A significant result at P < 0.01.**

**A significant result at P < 0.001.**
mutation and natural selection in somatic evolution. We have shown
evidence in a human neoplasm that both are important. The combi-
nation of clonal expansion and genetic instability predicts progression
to esophageal adenocarcinoma in Barrett’s neoplasms better than
either factor alone. Previous studies have shown that p53 LOH, aneuploidy,
and tetraploidy predict progression to esophageal adenocarcinoma (16, 17). Our results suggest that not just the presence but the size of
clones with these lesions predicts progression to esophageal ade-
nocarcinoma. Like aneuploidy (37–40) and tetraploidy (41), p53
lesions are associated with genetic instability (42–44). It seems
that an increasing number of genetically unstable cells favors the
evolution of a malignant clone. The presence of instability may
increase the rate of generation of genetic variants and subsequent
selection of clones with an increased predisposition to cancer. This
is consistent with data showing greater diversity of chromosomal
variants in biopsies with p53 LOH or aneuploidy than in diploid
biopsies from Barrett’s esophagus.5

The size of a clone with a p16 lesion appears to predict cancer,
but the effect disappeared with stratification on p53 lesions and
was not observed in Barrett’s segments that did not have a p53
lesion. In view of the width of the confidence intervals and the
weakness of the effects of p16-deficient clones (Table 2), stratifi-
cation by p53 LOH may result in an apparently nonsignificant
effect of p16 deficient clones on cancer outcome simply through a
reduction in the power to distinguish an effect. The lack of statis-
tical significance in the interactions between predictors in our data,
thus, does not preclude the presence of such interactions. Sample
size limitations make it difficult to accurately quantify risks and to
detect interactions of the genetic lesions. Loss of p16 is associated
with large clonal expansions to the point that the p16-deficient
clone often fills the entire Barrett’s segment (18, 24). In addition,
large clones with p16 lesions tend to include a p53 lesion. Taken
together, this suggests that p16-deficient clones are risk factors, in
part, because they may carry p53 lesions as hitchhikers (24) and
thus increase the size of the genetically unstable clone. Alterna-
tively, the loss of p16 may be necessary to allow a clone to spread
laterally in the Barrett’s epithelium; in this case, p16 loss would be
a prerequisite for subsequent expansion of genetically unstable
clones with a selective advantage.

The debate over the relative importance of genetic instability and
clonal expansion has concerned both neoplastic progression and ini-
tiation (3, 4). Our present results address factors that determine
whether or not a neoplasm will progress to cancer, and, thus, they do
not speak to the initiation of the premalignant neoplasm. Future
studies of early neoplasms in Barrett’s esophagus may also be able to
provide evidence of the roles of genetic instability and clonal expan-
sion in neoplastic initiation.

The size of genetically unstable clones may be used for prognoses
in other neoplasms in which clone sizes may be estimated through the
analysis of multiple biopsies or flow cytometry. The fact that large,
genetically unstable clones are more likely to progress to cancer than
smaller clones implies that cancer prevention efforts might be focused
on reducing the size or constraining the growth of genetically unstable
clones. To modulate clonal expansion, we will need a better under-
standing of clonal competition in neoplasms (24) and methods to
manipulate that competition (45, 46).

Genetic instability and clonal expansion are clearly important fac-
tors in neoplastic progression. Previous studies have focused on the
role of one or the other of these factors. We have shown in a human
premalignant condition that the combination of both genetic instabil-
ity and clone size predict progression to cancer in a prospective cohort
study.

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