APC Mutations and Other Genetic and Epigenetic Changes in Colon Cancer

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Abstract

Relationships between adenomatous polyposis coli (APC) mutations, BRAF V600E mutations, and the CpG island methylator phenotype (CIMP) in colon cancer have not been explored. In addition, controversies exist about the proportion of tumors with APC mutations in the mutation cluster region (MCR); how commonly APC, Ki-ras, and p53 mutations occur in the same tumor; and whether APC mutations occur in sporadic microsatellite-unstable tumors. The APC gene was therefore sequenced in 90 colonic adenocarcinomas previously evaluated for CIMP, microsatellite instability, BRAF, Ki-ras, and p53. APC mutations were inversely related to BRAF mutations (P = 0.0003) and CIMP (P = 0.02) and directly related to p53 and Ki-ras mutations (P = 0.04). Slightly more than half of APC mutations occurred outside of the MCR, and frameshift mutations were more likely than nonsense mutations to occur in the MCR (21 of 28 versus 12 of 40, P = 0.0003). APC mutations were found in sporadic microsatellite-unstable tumors and were more likely to be frameshifts in short nucleotide repeats (P = 0.007). The occurrence of APC, Ki-ras, and p53 mutations together in the same tumor was uncommon (11.1%). In conclusion, an analysis restricted to the MCR will miss more than half of APC mutations as well as mischaracterize their mutational spectrum. The conventional wisdom that most colon cancers contain APC, Ki-ras, and p53 mutations is incorrect. Microsatellite instability may precede acquisition of APC mutations in sporadic microsatellite-unstable tumors. The relationships of APC mutations to other genetic and epigenetic alterations add to the already impressive genetic heterogeneity of colon cancer.


Introduction

Mutations in the adenomatous polyposis coli (APC) gene have been reported to be the most common acquired genetic change in sporadic colon cancer (1-3). Recent studies have identified new genetic and epigenetic changes in colon cancer, especially oncogenic mutations in BRAF, part of the Ras/Raf/MEK/ERK signal transduction pathway, and the CpG island methylator phenotype (CIMP; ref. 4). No studies, to date, have evaluated whether relationships exist between APC mutations and either CIMP or BRAF.

There are also controversies about APC mutations in colon cancer. One is whether or not an evaluation of the mutation cluster region (MCR), an area extending from codon 1,286 to codon 1,585 (5, 6), is sufficient for assessing APC mutations in colon cancer. Whereas one study found that nearly 80% of tumors with APC mutations had mutations in this region (7), others have reported percentages <60% (2, 8). In addition, the generally accepted paradigm of colon cancer tumorigenesis for the majority of tumors has been a stepwise progression in which APC mutations are followed by several other mutations, including alterations in Ki-ras and p53 (9). This has also been questioned, as tumors with concurrent Ki-ras and p53 mutations (10) or APC, Ki-ras, and p53 mutations (11) seem to be quite uncommon.

Finally, there is some controversy about whether APC mutations occur in sporadic microsatellite-unstable colon cancers; a subset of unstable tumors associated with hereditary nonpolyposis colon cancer (HNPCC) are known to harbor APC mutations (12), but the literature has been divided on whether these mutations are seen in sporadic unstable colon cancers (8, 13). Studies also have differed on whether the spectrum of APC mutations in microsatellite-unstable tumors differs from that seen in stable tumors (13, 14). An excess of frameshift mutations in short microsatellite repeats in unstable tumors would imply that these mutations developed after the onset of microsatellite instability. One problem with reconciling these previous studies on APC mutations in sporadic microsatellite-unstable tumors has been the difficulty in excluding the possibility that a given unstable tumor was actually associated with HNPCC, a subset of which, as noted above, is known to harbor APC mutations. Recent studies, however, have shown that sporadic microsatellite-unstable tumors commonly exhibit CIMP and that many harbor BRAF mutations, but these findings are not seen in HNPCC-associated unstable tumors (15-17). Unstable tumors with either of these findings can therefore be classified as sporadic with a fair degree of certainty, and the question of the occurrence and/or type of APC mutation in sporadic microsatellite-unstable tumors can be more confidently addressed.
Results

The clinicopathologic features of the sample population and their tumors are shown in Table 1. Although the numbers are small, no significant differences were seen with respect to age, gender, tumor site or tumor stage, and the presence or absence of APC mutations.

Sixty-eight deleterious (nonsense, out-of-frame insertion or deletion) APC mutations were observed in 55 of 90 (59.1%) colon cancers. Forty-two tumors had one APC mutation, and 13 had two. Three missense and five silent mutations also were observed; these were not scored as deleterious and were not included in subsequent analyses. The types of deleterious APC mutations are shown in Table 2. There was a slight predominance of nonsense over frameshift mutations (58.8 - 41.2%). Most of the nonsense mutations were transitions (67.5%) and most of these occurred at CpG dinucleotides (81.5%). All of the CpG transitions altered the code for one amino acid, arginine (CGA); all of these affected the coding strand. The one transversion mutation that affected a CpG dinucleotide occurred in the noncoding strand.

Slightly less than half (48.5%) of APC mutations occurred inside the MCR, with most of the other mutations occurring 5' to the MCR (Table 2). Thirty-one of the 55 (56.4%) tumors with APC mutations had at least one mutation in the MCR (data not shown in table). Twelve of 40 nonsense mutations occurred in the MCR. In contrast, 21 of the 28 frameshifts occurred in the MCR, and this difference was highly significant (P = 0.0003). Twelve of the 21 frameshifts occurred in short mononucleotide repeats (three or more) or dinucleotide repeats, and eight of these occurred in the MCR; however, these short nucleotide repeats are spread throughout the gene and are not concentrated in the MCR (data not shown). Only 3 of the 22 transition mutations at CpG dinucleotides occurred in the MCR, and this was significantly different than the location of the other types of nonsense mutations with respect to the MCR (P = 0.03).

APC Mutations and Subtypes of Tumors

APC mutations were seen in all genetically defined groups of tumors, including cancers that were microsatellite unstable and CIMP high (Table 3). Every tumor subtype, except CIMP high/BRAF mutant, had tumors with two deleterious APC mutations; overall, 14.4% (13 of 90) tumors had two deleterious mutations. Although the numbers were small, there was a significant difference (P = 0.01) in the presence of APC mutations among the four genetically defined groups of tumors, as microsatellite stable/CIMP high/BRAF wild-type and microsatellite stable/CIMP low had a higher percentage of APC mutations (66.7% and 71.5%, respectively) than microsatellite stable/CIMP high/BRAF mutant and microsatellite unstable/CIMP high (30% and 33.4%, respectively).

APC and Other Genetic and Epigenetic Changes

There were statistically significant inverse relationships between APC mutations and BRAF mutations and CIMP (P = 0.0003 and P = 0.02, respectively), significant direct relationships between APC mutations and Ki-ras and p53 mutations (P = 0.04), and a trend toward an inverse relationship between microsatellite instability and APC mutations (P = 0.05; Table 4). In a multivariate analysis (Table 5), only the inverse relationship with BRAF and the direct relationship with p53 (P = 0.02 for both) remained significant, although the odds ratio for Ki-ras-mutated tumors also having an APC mutation was close to 2.

Microsatellite Instability and Types of APC Mutations

Four of six APC mutations in unstable, CIMP-high tumors were frameshift mutations occurring in short mononucleotide (three or more) or dinucleotide repeats (Table 6). In contrast, only 8 of 62 mutations in microsatellite-stable tumors were frameshifts in repeats; this difference was statistically significant (P = 0.007).

Concurrence of APC, Ki-ras, and p53 Mutations

In microsatellite-stable, CIMP-low cancers, the most common grouping of mutations was p53 and APC; this was seen in 26.3% of tumors (Fig. 1). The next most common mutation grouping was a mutation only in the APC gene; this was seen in 17.5% of tumors. Ki-ras and APC mutations

Table 1. Clinicopathologic Features

<table>
<thead>
<tr>
<th>All tumors</th>
<th>No APC mutations, n (%)</th>
<th>1 APC mutation, n (%)</th>
<th>2 APC mutations, n (%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at selection (y)</td>
<td>10 (29)</td>
<td>13 (31)</td>
<td>5 (38)</td>
<td>0.77</td>
</tr>
<tr>
<td>&lt;65</td>
<td>65-70</td>
<td>71-79</td>
<td>Male</td>
<td>17 (49)</td>
</tr>
<tr>
<td>Female</td>
<td>18 (51)</td>
<td>21 (50)</td>
<td>6 (46)</td>
<td>0.98</td>
</tr>
<tr>
<td>Site</td>
<td>Proximal</td>
<td>Distal</td>
<td>American Joint Committee on Cancer stage</td>
<td>I</td>
</tr>
<tr>
<td>II</td>
<td>12 (34)</td>
<td>10 (24)</td>
<td>5 (38)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>8 (23)</td>
<td>16 (38)</td>
<td>5 (38)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>7 (20)</td>
<td>6 (14)</td>
<td>2 (15)</td>
<td></td>
</tr>
</tbody>
</table>

*χ² (mutation versus no mutation).
occurred in the same tumor in 14% of the cancers, whereas Ki-ras, p53, and APC mutations were only seen in 12.3%, which is the same percentage of tumors without mutations in any of these three genes. The percent of tumors with all three mutations is approximately what one would expect due to chance (12.3% versus 11.9%, respectively), and the distribution of tumors with each combination of mutations shown in Fig. 1 did not depart from that expected by chance [Pearson \( \chi^2 \) (7 degrees of freedom) = 3.81, \( P = 0.80 \)]. If all 90 tumors are considered, these three mutations occurred together in 11.1% of cancers. No tumor had a mutation in only \( p53 \) and Ki-ras.

**Discussion**

This study reaffirms that \( APC \) mutations are the most common acquired mutation in sporadic colon cancer, occurring in 60.1% of all tumors. This observed frequency is essentially identical to the 60% reported by Powell et al. (1) in another study in which the entire coding region of \( APC \) was sequenced. There is a MCR for \( APC \) with respect to sporadic colon cancer, as nearly 60% of the tumors had at least one mutation in a region that corresponds to \( \sim 10.5\% \) of coding region of the gene. Still, studies that only evaluate this region would incorrectly classify these tumors as nearly 60% of the tumors had at least one mutation in a region corresponding to the MCR. Thus, the excess of mutations in the MCR is mostly supplied by frame-shifts. Therefore, studies that restrict themselves to the MCR will not only miss a substantial proportion of \( APC \) mutations but will also misrepresent the mutational spectrum, overemphasizing frame-shifts and underemphasizing nonsense mutations, especially the very common C-to-T transition at CpG dinucleotides at arginine codons.

The common occurrence of C-to-T transitions at CpG dinucleotides has previously been noted (1). Their occurrence in one particular type of codon (i.e., the CGA for arginine) probably reflects the fact that this CpG site is the only one in which a single C-to-T transition is sufficient to convert the amino acid code to a stop. A similar predilection for C-to-T transition mutations at CpG dinucleotides within arginine codons has been noted in \( p53 \) and retinoblastoma gene mutations (18, 19) and has been ascribed to methylation of cytosine by endogenous methyltransferases with subsequent deamination to thymine. Whereas this type of mutation has traditionally been thought to be unrelated to exogenous factors, some studies have suggested that the 5-methylcytosine may also serve as a target for carcinogens (20-22).

Another point of interest is that these arginine mutations occurred almost entirely outside of the MCR (only one of these codons is in the MCR), whereas most of the frame-shifts occurred in the MCR. The other 18 nonsense mutations were split evenly between the MCR and the region 5’ to the MCR. Thus, the excess of mutations in the MCR is mostly supplied by frame-shifts. Therefore, studies that restrict themselves to the MCR will not only miss a substantial proportion of \( APC \) mutations but will also misrepresent the mutational spectrum, overemphasizing frame-shifts and underemphasizing nonsense mutations, especially the very common C-to-T transition at CpG sites.

\( APC \) mutations were seen in all of the genetically defined subgroups of colon cancer we evaluated, including

| Table 3. \( APC \) Mutations in Genetically Defined Subgroups of Colon Cancer |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Total, \( n \) (%) | Location of mutation | \( P \)       |
|                                 |                 | MCR, \( \% \) | Outside MCR, \( \% \) |
| Total                           | 68              | 33 (48.5) | 35 (51.5) |
| Effect of mutation              |                 |             |             |
| Nonsense                        |                 |             |             |
| Frameshift                      | 28 (41)         | 21 (64) | 7 (20) |
| Nonsense                        | 40 (59)         | 12 (36) | 28 (80) |
| Transition at Cpg dinucleotide  | 22 (55)         | 3 (25) | 19 (68) |
| Other transition                | 5 (13)          | 3 (25) | 2 (7) |
| Transversion at Cpg dinucleotide| 1 (3)           | 1 (8) | 0 (0) |
| Other transversion              | 12 (30)         | 5 (42) | 7 (25) |

NOTE: From 90 tumor samples from a population-based series; 35 tumors had no effect of mutation.

*\( \chi^2 \) value is by Fisher’s exact test comparing the various types of nonsense mutations with respect to the location of the mutation inside or outside of the MCR.

Abbreviations: MSS, microsatellite stable; MSI, microsatellite unstable; wt, wild-type; mut, mutant.

*\( P \) value is by Fisher’s exact test comparing the four genetically defined classes of tumors with respect to the presence or absence of \( APC \) mutations.
microsatellite-unstable, CIMP-high tumors. Previous literature on the lack of CIMP-high in HNPCC-associated tumors (15, 16) supports the notion that these tumors are sporadic. In addition, 11 of these 12 had hMLH1 methylation (including all four with APC mutations; data not shown) and eight had Braf V600E mutations (including one of the tumors with an APC mutation), factors which also are most consistent with sporadic unstable tumors (15-17). The individuals with APC mutations were 79, 78, 78, and 66 years old, ages that would be unusual for onset of colon cancer in HNPCC. Finally, in a previous study (23), 9 of the 12 individuals with unstable, CIMP-high tumors were evaluated for germ line mutations in hMLH1 and hMSH2, the most commonly mutated mismatch repair genes in HNPCC. No germ line mutations were seen in these nine individuals (including three of the individuals whose tumors harbored APC mutations). Whereas APC mutations are known to occur in a subset of HNPCC-associated unstable tumors (12), their presence in sporadic unstable tumors has been controversial (8, 13), especially because it had been very difficult to completely exclude a germ line HNPCC mutation. Using our surrogate markers of CIMP-high, Braf mutations and hMLH1 methylation (and, in many cases, evaluation of the germ line itself for mismatch repair gene mutations), we can be fairly certain that this subset of tumors is sporadic and that some do harbor APC mutations. Nevertheless, our data also show a nearly significant inverse relationship between APC mutations and microsatellite instability, suggesting that both APC-driven and non–APC-driven pathways to sporadic unstable tumors exist. Two studies (24, 25) have also shown an inverse relationship between APC mutations and microsatellite instability, although in neither study was the complete gene evaluated.

There was a significant difference in the type of APC mutation with respect to microsatellite instability as well, as sporadic unstable tumors were characterized by frameshift mutations in short nucleotide repeats, a mutational signature of microsatellite instability. This implies that microsatellite instability preceded the development of APC mutations in these tumors, a finding in contrast to a previous study (14) but in agreement with another (13). Although this difference was statistically significant, it is based on small numbers of mutations and small numbers of unstable tumors. However, the data are consistent with what is known about the effect of mismatch repair deficiency on the replication of short nucleotide repeats. If verified by larger studies, this could have important implications for tumorigenic pathways. Because APC mutations are thought to be the initial mutation in most adenomatous polyps (1), the development of microsatellite instability before APC mutations would imply that some sporadic unstable cancers might develop from nonadenomatous polyps, perhaps the subset of hyperplastic polyps that exhibit Braf mutations and methylation, as in the so-called “serrated pathway” (15).

Statistically significant inverse relationships were seen between APC and Braf mutational and APC and CIMP, and direct relationships were seen between APC and p53 mutations and APC and K-ras mutations. In a multivariate analysis, however, only the relationships with Braf and p53 remained significant. It is possible that with larger numbers of tumors, the relationship with K-ras might also become significant in a multivariate analysis, as the odds ratio for an APC mutation was close to 2. However, it is questionable whether larger numbers would change the results for CIMP or microsatellite instability, as the odds ratios associated with these genetic and epigenetic changes were very close to 1. For the case of microsatellite instability, this implies that the inverse relationship seen in this study and other studies could be a function of the relatively high frequency of Braf mutations in these tumors; indeed, a significant univariable inverse relationship between APC and Braf was seen in an evaluation of microsatellite-stable tumors by themselves (*P = 0.03; data not shown). We have recently observed a similar phenomenon in the relationship of smoking to colon cancer, as the previous observation of a relationship between smoking and microsatellite instability (26) turned out to be explained by a relationship between smoking and Braf and CIMP (27). It should also be noted that the relationship between APC mutations and Braf may reflect the very high degree of CpG island methylation seen in Braf-mutated tumors (27), and that other measures of CIMP, which identify more heavily methylated tumors, may show stronger relationships (28).

APC mutations are seen in most tumors with K-ras or p53 mutations. However, even the groups with K-ras and p53 mutations do not always have APC mutations, and it is actually quite uncommon to see all three mutations in one tumor. Thus, the most common subset of colon tumors (i.e., microsatellite stable and CIMP low) only rarely show all three mutations. One would predict that this subset of tumors also would be the most likely of the groups we studied to show the presence of

| Table 5. Multivariate Analysis of APC Mutations with Other Molecular Alterations |
|-----------------|-----------------|-----------------|
| **OR for APC mutation (95% CI)** | **P** |
| **Braf** mut vs wt | 0.135 (0.025-0.720) | 0.02 |
| CIMP high vs low | 0.926 (0.233-3.673) | 0.91 |
| K-ras mut vs wt | 1.995 (0.607-6.554) | 0.26 |
| p53 mut vs wt | 3.513 (1.212-10.184) | 0.02 |
| Microsatellite unstable vs stable | 1.038 (0.175-6.146) | 0.97 |

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
TABLE 6. Frameshift Mutations in Microsatellite-Unstable, CIMP-High Tumors

<table>
<thead>
<tr>
<th>SID</th>
<th>Nucleotide position</th>
<th>Original sequence</th>
<th>Mutated sequence</th>
<th>Mutation type</th>
<th>Mutation in repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,060</td>
<td>4,092</td>
<td>GAGAAAGAGGCAGAAAAACTAT</td>
<td>GAGAAAG__GCAGAAAAAAACTAT</td>
<td>2 bp AG del in an AGAG repeat</td>
<td>1,746</td>
</tr>
<tr>
<td>4,655</td>
<td>5,234</td>
<td>GTGTGAAAAAGATAATG</td>
<td>GTGTGAAAA_AGATAATG</td>
<td>1 bp A del in a AAAA repeat</td>
<td>1,746</td>
</tr>
<tr>
<td>4,386</td>
<td>4,691</td>
<td>CCCTCCAAAAGTGGTGCT</td>
<td>CCCTCCAAA_GTGGTGCT</td>
<td>2 bp TA del which does not occur in a repeat</td>
<td>1,746</td>
</tr>
</tbody>
</table>

Abbreviation: SID, study identification.

All three mutations. Microsatellite-unstable tumors have been shown to have significantly fewer p53 and Ki-ras mutations (ref. 29; as well as a trend toward decreased APC mutations as shown in the present study). CIMP-high and BRAF-mutated tumors were also shown to have fewer APC mutations in the current study. The relative rarity of concurrence of these three mutations even in stable/CIMP-low tumors contradicts the prevailing notion of the accumulation of certain genetic events (i.e., APC, Ki-ras, and p53 mutations) in most colon cancers (9). Instead, this combination of mutations is the exception rather than the rule. A previous study came to much the same conclusion, but it restricted its analysis to the MCR of APC (11). As we have shown in this study, 51.4% of APC mutations occur outside of the MCR (including 44% of all tumors with at least one APC mutation), and so here we present a more definitive analysis of the relative lack of concurrence of APC mutations with other mutations.

Some APC mutations were undoubtedly missed by this study. Deletions of the entire gene (as seen by loss of heterozygosity), for example, would not have been detected by our sequencing method. Loss of heterozygosity is uncommon in microsatellite-unstable tumors (30, 31), however, and so the lack of deletion detection would probably not have affected our findings about the presence or timing of APC mutations in these tumors. Nor would it affect our findings about the frequency of mutations within the MCR and the mutational spectrum of mutations inside and outside of this region. Finally, biallelic deletion of APC is not commonly seen in colon cancer (2), and so detecting the coding region mutation is probably adequate in most cases to classify tumors with respect to their APC status. Promoter methylation, another potential way to inactivate a tumor suppressor gene by inhibiting transcription, was also not evaluated in this study. However, the significance of APC promoter methylation as a means toward inactivating this gene has been questioned (32). Finally, oncogenic muta-

In summary, and especially if replicated by other studies, our study affirms the importance of APC in all known tumorigenic pathways to sporadic colon cancer and underscores the impressive genetic heterogeneity of these pathways. It is likely that the APC mutational status and type of mutation in genetically defined subtypes of cancer will have implications for molecular epidemiologic and clinicopathologic studies and should be considered in such evaluations to provide a more complete understanding of this remarkably heterogeneous disease.

Materials and Methods

Sample Selection

Tumors were chosen from a previous population-based study (4) to reflect known genetic subtypes of colon cancer: microsatellite stable/BRAF V600E mutant (10 tumors), microsatellite stable/CIMP high/BRAF wild-type (12 tumors), microsatellite stable/CIMP low (56 tumors), and microsatellite unstable/CIMP high (12 tumors). Microsatellite-unstable/CIMP-low tumors were not used to avoid the possibility of inclusion of individuals with HNPCC. The number of tumors in each group approximates what we have previously seen in this population (4) with the exception of the microsatellite-stable/BRAF-mutant group; this is about twice as large as would be expected in this set of 90 tumors, and was chosen to increase our power to detect possible associations between APC mutations and BRAF mutations in microsatellite-stable tumors.

Primer Design and PCR Conditions

All PCRs were done in 12-μL reaction volumes in a 96-well format GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) using AmpliTaq Gold DNA polymerase and following standard protocols. Thermocycling profile included 10 min at 95°C, followed by 30 cycles of 10-s denaturation at 95°C, 10-s annealing, and 20-s extension at 72°C. Primer sequences, annealing temperatures, and MgCl2 concentrations specific to individual amplicons are available upon request. For the nested PCR, a 2-μL aliquot of the external PCR product was diluted 25-fold and 2 μL of the diluted product were used as template for the internal PCR amplification. Two microliters of the internal PCR products were visualized in 2% agarose gels in 1× Tris-acetate-EDTA to assess PCR product size and quality.

A panel of 38 amplicons were used to cover the entire coding region of APC. Evaluable sequence data were obtainable from 99.9% of the 3,420 amplicons. All sequences with the exception of exon 4 are bidirectional. The exon 4 amplicon provided clean sequence in the reverse direction, but a nucleotide repeat adjacent to the 5′ exon boundary prevented sequencing in the forward direction. All mutations were verified using original template DNA in separate PCR and sequencing reactions.

Sequencing and Sequence Analysis

PCR products were prepared for DNA sequencing using the ExoSAP-IT method (U.S. Biochemical Corp, Cleveland, OH) and sequenced in a 3730 96-capillary sequencing instrument (Applied Biosystems) using standard procedures. Sequences were assembled and analyzed using SeqScape (version 2.5; Applied Biosystems).

Data Analysis

Mutations were categorized according to type of mutation. Nonsense and out-of-frame insertion or deletion mutations were classified as deleterious mutations; missense and silent mutations were not considered deleterious and were not included in data analysis. We evaluated differences in the distribution of type of deleterious APC mutations by location in the gene, as well as associations between deleterious APC mutations and other tumor mutations, using contingency tables and either Pearson χ² or Fisher’s exact test. A multivariate logistic regression including BRAF, CIMP, Ki-ras, p53, and microsatellite instability as covariates was also done. As mentioned above, evaluable sequence data were obtainable from 99.9% of the 3,420 amplicons for APC. Complete results for the BRAF V600E mutation, CIMP, and microsatellite instability were available for all 90 tumors, and results for Ki-ras and p53 were available for 88 tumors. The expected joint distribution of Ki-ras, p53, and APC mutations in microsatellite-stable, CIMP-low tumors was calculated from the frequencies of each and compared with the observed distribution using Pearson χ². All statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, NC).

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References

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