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Review

Cancer Research

Cooperation and Antagonism among Cancer Genes: The Renal Cancer Paradigm

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Abstract

It is poorly understood how driver mutations in cancer genes work together to promote tumor development. Renal cell carcinoma (RCC) offers a unique opportunity to study complex relationships among cancer genes. The four most commonly mutated genes in RCC of clear-cell type (the most common type) are two-hit tumor suppressor genes, and they cluster in a 43-Mb region on chromosome 3p that is deleted in approximately 90% of tumors: *VHL* (mutated in ∼80%), *PBRM1* (∼50%), *BAP1* (∼15%), and *SETD2* (∼15%). Meta-analyses that we conducted show that mutations in *PBRM1* and *SETD2* co-occur in tumors at a frequency higher than expected by chance alone, indicating that these mutations may cooperate in tumorigenesis. In contrast, consistent with our previous results, mutations in *PBRM1* and *BAP1* tend to be mutually exclusive. Mutation exclusivity analyses (often confounded by lack of statistical power) raise the possibility of functional redundancy. However, mutation exclusivity may indicate negative genetic interactions, as proposed herein for *PBRM1* and *BAP1*, and mutations in these genes define RCC with different pathologic features, gene expression profiles, and outcomes. Negative genetic interactions among cancer genes point toward broader context dependencies of cancer gene action beyond tissue dependencies. An enhanced understanding of cancer gene dependencies may help to unravel vulnerabilities that can be exploited therapeutically. *Cancer Res; 73(14); 4173–9. ©2013 AACR.*

Introduction

Cancer research has been revolutionized by massively parallel sequencing. Up to 5% of protein-coding genes are potential cancer genes implicated in the development of the disease (1, 2). Many novel cancer genes have been discovered, providing inroads into the molecular pathogenesis of tumors and setting a foundation for a molecular classification of cancer (3, 4).

Cancer drivers may be distinguished from passenger genes by their mutation at frequencies higher than expected by chance alone. Oncogene drivers, which are typically activated by mutation and tend to be dominant, may be recognized by the presence of recurrent missense mutations at a limited number of residues. However, multiple residues may be targeted by mutations that disrupt autoinhibitory domains (as in mTOR). In contrast, tumor suppressor genes, which are inactivated by mutation and are typically recessive, may be disrupted by a variety of alterations, including insertions, deletions, nonsense, missense, and splice-site mutations. Missense mutations in tumor suppressor genes are often used to identify domains important for function, but these analyses are confounded by mutations disrupting secondary or tertiary structure and causing protein instability. Typically, one allele of a tumor suppressor gene is disrupted by a focal mutation and the other is lost as part of a large deletion, which results in loss of heterozygosity (LOH).

Further complexity arises from mutation heterogeneity in tumors (5), which results from plasticity and clonal evolution (6). According to their prevalence, somatic mutations may be divided into ubiquitous, shared, and private. Ubiquitous mutations (present in all tumor cells) encompass truncal driver events. However, not every ubiquitous mutation is a driver mutation (preexisting mutations in the lineage giving rise to the initial tumor clone make up ubiquitous passengers; ref. 7). Conversely, not every driver mutation may be ubiquitous, and mutations conferring invasive or metastatic potential may be found in only a subset of cells in primary tumors. This complexity can be advantageous. It can be harnessed to identify driver genes that tend to be mutated early, ubiquitous drivers. Pathways deregulated by ubiquitous drivers are optimal targets for drug therapies that seek to affect all tumor cells.

One of the challenges hampering mutation detection in solid tumors is contamination by normal stroma. DNA from stromal cells dilutes tumor DNA and reduces the sensitivity for mutation discovery. In addition, contamination makes it difficult to assess how homogeneously a given mutation is present across

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tumor cells in a sample. The prevalence of a mutation can be estimated by the mutant allele ratio (MAR), the fraction of the mutant over the mutant plus wild-type sequences for a given mutation (8). A heterozygous mutation would be expected to have a MAR of 0.5. Lower MARs may indicate that the mutation is present in only a subset of tumor cells in a sample (not a ubiquitous mutation), but this assessment is precluded by stromal contamination (8). Mutation sensitivity and MAR accuracy can be improved by the analysis of tumorgrafts, human tumors implanted in mice (8). In tumorgrafts, the stroma is replaced by the host and tumorgrafts preserve the characteristics of human tumors (9). However, studies in tumorgrafts rely on the ability to specifically query the human genome. Despite this limitation, MAR analyses in tumorgrafts can be instrumental to determine the prevalence of a mutation in a sample. Accurate MARs are helpful in a variety of other contexts. When mutations are found in areas of copy neutral LOH, MARs can show whether the mutation is homozygous (8). In addition, for mutations in areas of amplification, whether the mutant or the wild-type sequence is amplified can be determined with accurate MARs (8).

A comprehensive list of mutations in a tumor, together with an understanding of their prevalence and functional significance, should pave the way for better analyses of genetic interactions among cancer genes.

Evaluating Functional Relationships among Cancer Genes

Unraveling relationships among genes driving tumorigenesis is a challenge and represents the next frontier. A form of genetic interaction commonly reported is mutation exclusivity. Exclusivity is predicated of genes that are mutated in a particular tumor type, but not simultaneously. Often, mutation exclusivity is interpreted as evidence of functional redundancy. This is illustrated, for instance, by mutations in p16, D-type cyclins, CDK4, and retinoblastoma, which tend to be exclusive and disrupt the same cell-cycle regulatory pathway.

Mutation exclusivity is frequently misinterpreted owing to insufficient statistical power. As an example, when two genes are mutated at a frequency of 5%, the number of tumors required to show that a lack of mutation co-occurrence is due to a genetic interaction (as opposed to chance alone, after all, each gene is mutated in only 5% of the tumors) is 1,330. Thus, mutation exclusivity analyses may require meta-analyses of multiple studies, particularly when the interactions involve genes mutated at low frequencies.

Mutations and physical location

Another level of complexity is introduced by the physical location of cancer genes in chromosomes. In fact, the architecture of amplifications and deletions in tumors may be far more informative than previously appreciated. Traditionally, amplifications and deletions have typically been thought to be driven by a single gene, but more than one gene may be implicated in each region (see below). This has important methodological implications, as the hunt for cancer genes may need to be redirected toward genes flanking a common region of amplification or deletion, rather than those at the center.

Renal Cancer, a Paradigm

Renal cell carcinoma (RCC) offers a unique opportunity to study complex relationships among cancer genes. RCC is classified histologically into several types, including clear-cell RCC (ccRCC), the most common type. Positional cloning studies of kindreds with a ccRCC predisposition syndrome, von Hippel–Lindau (VHL), led to the identification of the eponymic gene, *VHL* (10). Subsequently, *VHL* was found to be frequently mutated in sporadic ccRCC (11). *VHL* is mutated in approximately 80% of sporadic ccRCC and is inactivated by methylation in an additional 10% (12, 13). *VHL* is rarely mutated in other sporadic tumors (14), suggesting that the tumor suppressor function of *VHL* is limited to a small number of cell types. The *VHL* gene encodes the substrate recognition subunit of an E3–ubiquitin ligase complex that triggers the degradation of, among others, the α-subunit of hypoxia-inducible factor (HIF) transcription factors (15). The *VHL* gene is on 3p25.3, and for many years it was thought to explain LOH at 3p in ccRCC.

Interestingly, four tumor suppressor genes have been identified in a relatively small (43 Mb) region on 3p. These genes are: *VHL*, SET domain containing 2 (*SETD2*; ref. 16), BRCA1-associated protein-1 (*BAP1*; refs. 8, 17), and Polybromo 1 (*PBRM1*; ref. 18). Each functions as a classical two-hit tumor suppressor gene, and an analysis we conducted of previously published data (8, 19) shows that this region is lost in 90% of sporadic ccRCC (Fig. 1). *PBRM1* is mutated in approximately 50% of ccRCC (18) and encodes BAF180 (herein referred to as PBRM1), the chromatin targeting subunit of a SWI/SNF nucleosome-remodeling complex. Both *SETD2* and *BAP1* are mutated in approximately 15% of ccRCC. *SETD2* is a histone H3K36 methyltransferase (20) specifically implicated in trimethylation (21). *BAP1* is a nuclear deubiquitinase (22, 23), and while substrates have been identified in *Drosophila* (24) and mammals (25–28), the relevant substrate(s) in RCC remain unknown (8).

Cooperation among tumor suppressor genes on 3p

We performed meta-analyses to test for genetic interactions among 3p genes. Given the mutation frequencies of the different genes, we used *PBRM1* as a reference. *PBRM1* is mutated at a sufficiently high frequency to be able to draw conclusions and, yet, not uniformly as *VHL*. We identified studies (or datasets) reporting mutations in *PBRM1* together with either *SETD2* or *BAP1*. Adding to the challenge of uncovering genetic interactions (as determined by the frequency of *VHL* mutations detected), mutation sensitivity was seemingly low across all of the studies available (16, 17, 29).

The Sanger Institute sequenced 348 ccRCC for both *PBRM1* and *SETD2*. One hundred and eleven tumors were found to be solely mutated for *PBRM1*, and 7 were found solely mutant for *SETD2* (18). Given the individual mutation rates for *PBRM1* and *SETD2*, 5 tumors were expected to have mutations in both

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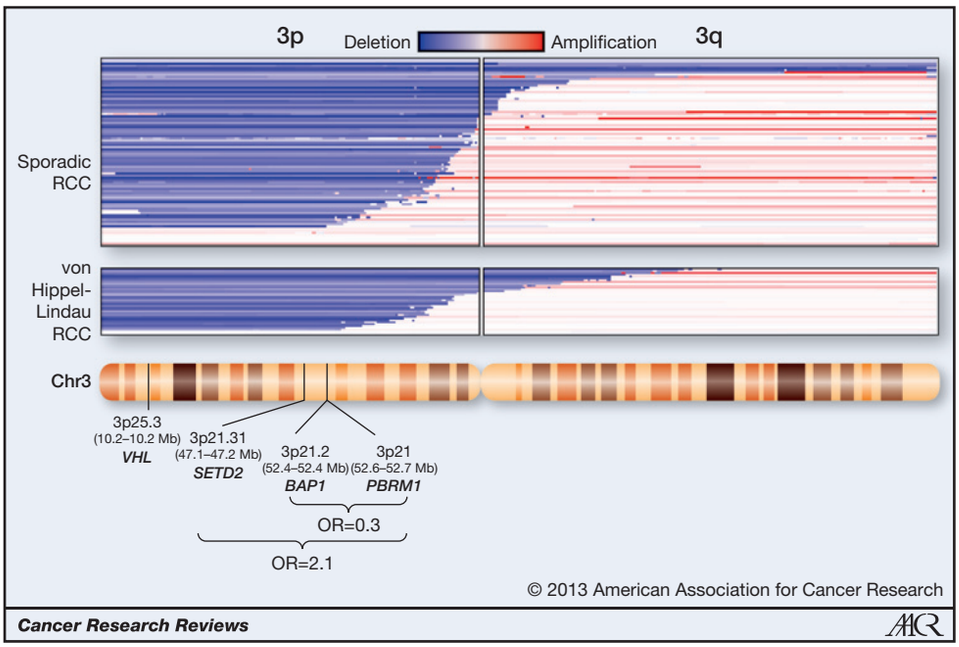

Figure 1. Schematic of chromosome 3 with the estimated position of *VHL*, *SETD2*, *BAP1*, and *PBRM1* genes and corresponding DNA copy number alterations in sporadic and familial (von Hippel–Lindau syndrome) ccRCC. Chromosome 3 ideogram [NCBI build 37.5 (hg19)] with superimposed copy number analyses of primary ccRCC tumors from GSE14994 and GSE25540. ORs for the finding of simultaneous mutations among the indicated genes are shown.

genes, but 8 were found ($P = 0.16$; Table 1). In a study conducted by Guo and colleagues involving 98 ccRCC, 18 tumors were solely mutant for *PBRM1* and 1 was solely mutant for *SETD2* (17). Again, given the mutation rates for *PBRM1* and *SETD2*, 1 tumor may have been expected to have mutations in both genes, but 3 were found ($P = 0.03$; Table 1). Hakimi and colleagues analyzed 185 ccRCC; 48 were found solely mutated for *PBRM1* and 8 solely mutated for *SETD2*. Given these mutation frequencies, 4 double-mutant tumors were expected, but 6 were found ($P = 0.24$; Table 1; ref. 29). Finally, The Cancer Genome Atlas consortium (TCGA; ref. 30) released results of 293 tumors; 90 tumors were found with mutations in *PBRM1* and 17 with mutations in *SETD2*. Twelve tumors were expected to have mutations in both *PBRM1* and *SETD2*, but 16 were found ($P = 0.13$; Table 1). Across all the studies, the number of tumors with mutations in both *PBRM1* and *SETD2* exceeded the number expected by chance alone. However, this difference reached statistical significance only in the study by Guo and colleagues (17). Nonetheless, when considered together, among 924 ccRCC, there were 267 tumors with mutations only in *PBRM1*, 33 with mutations only in *SETD2*, and 33 with mutations in both *SETD2* and *PBRM1* (Table 1). The number of tumors expected to have mutations in both genes by chance alone was 21, and 33 were found. Although the difference in absolute numbers is small, it represents an increase by one third, and the probability that this finding occurred by chance alone is 0.003. Overall, the frequency of mutations in *SETD2* was 2-fold higher for *PBRM1*-mutant tumors than wild-type tumors [odds ratio (OR), 2.1; 95% confidence interval (CI), 1.3–3.5].

Table 1. Cooperation between *SETD2* and *PBRM1* in ccRCC

Study	*n*	*PBRM1*	*SETD2*	*SETD2/PBRM1*	Expected double mutants	*P*	OR (95% CI)
Sanger Institute (16, 18)	348	111	7	8	5 (2–8)	0.16	2.3 (0.8–6.5)
Guo et al. (17)	98	18	1	3	1 (0–2)	**0.03**	12.7 (1.2 –129)
Hakimi et al. (29)	185	48	8	6	4 (2–7)	0.24	1.9 (0.6–5.8)
TCGA	293	90	17	16	12 (7–14)	0.13	1.8 (0.9–3.7)
Total	**924**	**267**	**33**	**33**	**21 (17–25)**	**0.003**	**2.1 (1.3–3.5)**

NOTE: The data presented here represent the number of tumors with specific mutations and expected frequencies. The range of expected double mutants was calculated on the basis of a hypergeometric distribution. Differences between actual and expected values were evaluated with a Fisher exact test. The Mantel–Haenszel test was used to integrate ORs from the different studies. Data on clear cell renal cell carcinoma (KIRC) were obtained from TCGA (January 2013 release). Data from the combination of these studies (the total) are highlighted in bold. Significant *P* values are also in bold.

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These results suggest that mutations in *PBRM1* and *SETD2* cooperate in renal tumorigenesis. They assume that mutations occur independently and in the same tumor cells, and functional studies will be required for confirmation. The biologic basis for this cooperation remains to be determined, but plausible models may be proposed on the basis of the function of these proteins, in particular, because both proteins converge on histones, one as writer of a histone mark (SETD2) and the other as a reader (PBRM1). Despite this cooperation, however, no differences in overall survival were found between patients with *PBRM1*-mutated tumors and those with tumors mutated in both *SETD2* and *PBRM1* (Supplementary Fig. S1).

Antagonism among tumor suppressor genes on 3p

We reported previously that mutations in *PBRM1* and *BAP1* are largely mutually exclusive (8), which stands in contrast to the findings from meta-analyses reported herein of *PBRM1* and *SETD2*. Among 176 ccRCC we analyzed, we found 89 tumors with mutations solely in *PBRM1* and 21 with mutations solely in *BAP1*. By chance, 13 tumors would have been expected to have mutations in both genes, but only 3 tumors were found. The probability that this observation was by chance alone was very low ($P = 0.00003$; ref. 8).

Guo and colleagues and Hakimi and colleagues have also reported *BAP1* and *PBRM1* mutations in ccRCC (17, 29). In both studies, the sensitivity for mutation detection was seemingly low, and consequently statistical power was insufficient. However, in both instances, fewer tumors were found with simultaneous mutations in both genes than were expected by chance alone (Table 2). Similarly, in data from TCGA, there was an under-representation of tumors with mutations in both *BAP1* and *PBRM1* (Table 2). In the TCGA study, which is the largest, among 293 tumors, there were 101 with mutations solely in *PBRM1* (independently of *SETD2*) and 22 with mutations solely in *BAP1*. Given the relative frequencies of tumors individually mutated for *BAP1* and *PBRM1*, 10 tumors would have been expected to have mutations in both genes, but only 5 were found ($P = 0.058$; Table 2). In the aggregate, these three studies evaluated 576 tumors and among them there were 175 tumors with mutations solely in *PBRM1* and 40 with mutations solely in *BAP1*. Considered together, 14 tumors would have been expected with mutations in both *BAP1* and *PBRM1*, but only 6 were found, and the *P* value was significant ($P = 0.004$). Thus, the odds of having a *BAP1* mutation in *PBRM1*-mutant tumors are one third of those for wild-type tumors (OR, 0.29; 95% CI, 0.12–0.70). Although we cannot exclude the possibility that mutation co-occurrence rates may be affected by epigenetic changes (ref. 31; or other factors), these data suggest that simultaneous mutations in *BAP1* and *PBRM1* are negatively selected for in ccRCC.

Mutation exclusivity is often interpreted to indicate functional redundancy. However, differences in pathologic features, gene expression, and outcomes between tumors with *BAP1* and *PBRM1* mutations suggest that *BAP1* and *PBRM1* are not functionally redundant. *BAP1*-mutant tumors tend to be of high grade, whereas tumors exclusively mutated for *PBRM1* are typically of low grade (8). *BAP1*-mutant tumors, but not *PBRM1*-mutant tumors, are associated with activation of the mTORC1 pathway (8, 32), a critical pathway in ccRCC (33). *BAP1*- and *PBRM1*-mutant tumors are associated with different gene expression signatures. We analyzed 308 ccRCC from the TCGA that had RNA-Seq data available and found that, when compared with the rest, 3,250 genes distinguished the *BAP1*-mutant group ($n = 20$) and 2,235 genes distinguished the *PBRM1*-mutant group ($n = 66$; ref. 32). In contrast, when groups of tumors were assembled arbitrarily, the number of genes that distinguished these random groups from the rest was less than 200. The differences in the number of genes associated with the *BAP1*- and *PBRM1*-mutant groups versus the random groups was highly statistically significant ($P < 0.0001$; ref. 32). These data indicate that the signatures identified are highly specific. The *BAP1*- and *PBRM1*-mutant signatures did not overlap

Table 2. Antagonism between *BAP1* and *PBRM1* in ccRCC

Study	*n*	*PBRM1*	*BAP1*	*BAP1/PBRM1*	Expected double mutants	*P*	OR (95% CI)
Peña-Llopis et al. (8)	176	89	21	3	13 (9–16)	**0.00003**	0.10 (0.03–0.35)
Guo et al. (17)	98	21	8	0	2 (0–4)	0.20	0.19 (0.01–3.43)
Hakimi et al. (29)	185	53	10	1	3 (1–5)	0.18	0.23 (0.03–1.83)
TCGA	293	101	22	5	10 (7–13)	0.058	0.37 (0.14–1.01)
Total	**576**	**175**	**40**	**6**	**14 (11–18)**	**0.004**	**0.29 (0.12–0.70)**

NOTE: Number of tumors with specific mutations and expected frequencies. The range of expected double mutants was calculated on the basis of a hypergeometric distribution. Differences between actual and expected values were evaluated with a Fisher exact test. The Mantel–Haenszel test was used to integrate ORs from the different studies. A fixed 0.5 correction was used to find the OR when there was a frequency of 0. Data on clear cell kidney and renal cell carcinoma (KIRC) were obtained from TCGA (January 2013 release). Note that differences in the number of tumors with mutations in *PBRM1* shown in Table 1 reflect how tumors are divided according to *SETD2* or *BAP1* status. Data from the combination of the three studies (the total) are highlighted in bold. Significant *P* values are also in bold.

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beyond what was expected by chance alone, indicating that they were different (32). *BAP1*-mutant tumors and *PBRM1*-mutant tumors are also associated with different outcomes in patients (32). Although the median overall survival for patients with *BAP1*-mutant tumors is 4.6 years (95% CI, 2.1–7.2), for patients with *PBRM1*-mutant tumors, the median survival is 10.6 years (95% CI, 9.8–11.5; HR, 2.7 and 95% CI, 0.99–7.6; $P = 0.044$; ref. 32).

Taken together, differences in pathology, gene expression, and outcomes strongly suggest that the BAP1 and PBRM1 proteins regulate different processes. Thus, the observation that *BAP1* and *PBRM1* mutations co-occur in tumors at a frequency lower than expected suggests that, even within a tumor type, a context dependency of tumor suppressor function may exist. Although in some contexts mutations may be tolerated and be advantageous, the same mutations may not be tolerated in other contexts.

The context dependency of tumor suppressor function fits well with the empiric observation that genes exert their tumorigenic properties in a contextual, tissue-dependent manner. This is illustrated in familial cancer syndromes, in which a germline mutation (typically in a tumor suppressor gene) predisposes to a limited spectrum of tumors. Thus, despite the presence of the mutation in all diploid cells, tumors arise in a limited number of tissues. Other factors could help to explain the limited tissue repertoire, including differences in rates of mutation of the remaining allele across cell types. Nonetheless, a limited tumor spectrum is also observed in familial cancer syndromes resulting from germline mutations in oncogenes, such as *RET*, which are not associated with a mutation of the second allele. Other examples of contextual effects are provided by the overexpression of certain oncogenes, which, depending on the cellular context, may induce senescence or proliferation (34). We conjecture that contextual differences in cancer gene action extend beyond tissue boundaries such that, even within a specific tumor type, there may be permissive and nonpermissive contexts (dictated perhaps by other mutations). Thus, tumors may be viewed as an evolving set of conditional dependencies, which, if understood, may uncover vulnerabilities that could be exploited therapeutically.

A model for ccRCC development

We propose that ccRCC development evolves along two different paths. Following a *VHL* mutation, which is an early event (35, 36), and the loss of 3p, which is frequently observed (Fig. 1), mutations in the remaining *PBRM1* or *BAP1* allele may lead to tumors with different characteristics. Thus, tumor aggressiveness may be programmed early during ccRCC development. This model may explain why, despite the discovery of the *VHL* gene in 1993 (10), a mouse model of ccRCC does not exist today. Interestingly, although the *VHL* gene is linked to *PBRM1* and *BAP1* on the same 3p arm in humans, *Vhl* is on a different chromosome from that of *Pbrm1* and *Bap1* in the mouse. Thus, loss of heterozygosity of the *Vhl* region in the mouse would not simultaneously inactivate one copy of *Pbrm1* and *Bap1*. If this model is correct, ccRCC should develop in mice with simultaneous inactivation of *Vhl* and either *Pbrm1* or *Bap1* genes, a testable hypothesis currently under evaluation.

Thus, the physical location of cancer genes in the genome may dictate the spectrum of tumors to which a particular species may be predisposed. Thus, in some species, a deletion may eliminate a combination of tumor suppressor genes conducive to tumor development, but if the genes are not collinear in another species, the species may be protected from the corresponding tumor type.

The physical location of tumor suppressor genes may also have implications within a species. For example, the type of second hit mutation observed may depend on whether there are neighboring tumor suppressor genes that function as such in the specific tissue. For instance, *BAP1* is mutated in mesothelioma (37, 38), ccRCC (8, 17), and uveal melanoma (39–41). However, the "second-hit" mutation may be different in the three tissues. In mesothelioma, focal mutations may inactivate the second allele (38), whereas in ccRCC the second allele is typically inactivated by loss of 3p (Fig. 1), and in metastatic uveal melanoma through whole chromosome 3 loss (39). These data are consistent with the notion that *VHL* (as well as *SETD2* and *PBRM1*) may not function as a tumor suppressor gene in mesothelioma, in contrast with ccRCC. The data also suggest the existence of other tumor suppressor genes in metastatic uveal melanoma in 3q. We speculate that differences in the type of second-hit mutation across tissues illustrate tissue-specific differences in tumor suppressor gene activities and tumor suppressor gene cooperation.

The type of second-hit mutation may also depend on noncancer genes. Noncancer genes may be subject to dosage effects, and these effects may be context or tissue specific. Thus, a large deletion may be poorly tolerated in some tumor types as it may uncover tissue-specific haploinsufficient genes, diminishing, thereby, the fitness of the tumor cell.

Conclusions

Improved functional annotation of mutations in cancer genes and an understanding of mutant allele ratios and mutation prevalence in tumors should facilitate the development of genetic interaction maps. To uncover the full spectrum of genetic interactions among cancer genes, adequate statistical power is necessary and meta-analyses may be required. Large deletions in tumors may be driven by the loss of more than one tumor suppressor gene, and syntenic differences may explain differential tumor predisposition across species. Together with the notion that tumor suppressor genes function as such in a tissue-restricted manner, the physical location of a gene may explain the type of second-hit mutation and the architecture of deletions across different tumor types. Understanding genetic interactions among driver genes and context dependencies of oncogenic (or tumor suppressor) action, which extend beyond tissue boundaries, may expose vulnerabilities that could be exploited therapeutically.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Brugarolas
Development of methodology: S. Peña-Llopis, J. Brugarolas
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Peña-Llopis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Peña-Llopis, A. Christie, X-J. Xie, J. Brugarolas
Figure preparation: S. Peña-Llopis
Writing, review, and/or revision of the manuscript: J. Brugarolas
Study supervision: J. Brugarolas

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