USING SOMATIC MUTATION DATA TO TEST TUMORS FOR CLONAL RELATEDNESS[1]

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A major challenge for cancer pathologists is to determine whether a new tumor in a patient with cancer is a metastasis or an independent occurrence of the disease. In recent years numerous studies have evaluated pairs of tumor specimens to examine the similarity of the somatic characteristics of the tumors and to test for clonal relatedness. As the landscape of mutation testing has evolved, a number of statistical methods for determining clonality have developed, notably for comparing losses of heterozygosity at candidate markers, and for comparing copy number profiles. Increasingly tumors are being evaluated for point mutations in panels of candidate genes using gene sequencing technologies. Comparison of the mutational profiles of pairs of tumors presents unusual methodological challenges: mutations at some loci are much more common than others; knowledge of the marginal mutation probabilities is scanty for most loci at which mutations might occur; the sample space of potential mutational profiles is vast. We examine this problem and propose a test for clonal relatedness of a pair of tumors from a single patient. Using simulations, its properties are shown to be promising. The method is illustrated using several examples from the literature.

1. Introduction. One of the major routine tasks of cancer pathologists is to determine if a new tumor identified in a patient with cancer is a metastasis of the original primary tumor or a completely new, independent occurrence of the disease. Traditionally this diagnosis has been accomplished by comparing the gross histologic features of the tumor cells, but in recent years evidence from genetic markers has increasingly come to inform this decision. At the molecular level the DNA of individual tumors is characterized by many somatic changes, including mutations in individual genes and losses or gains of large segments of DNA (copy number changes). Two tumors that originally evolved from the same "clone" of cancer cells will thus possess some somatic changes that are identical. These identical changes will be present in both the primary tumor and the metastasis that is seeded by the primary. In contrast, any similarities in mutational or copy number profiles of pairs of independently occurring cancers must occur by chance. Consequently, comparison of the DNA profiles for the extent of similarities in the

patterns of somatic changes is a powerful strategy for determining the diagnosis of a new tumor as independent or as a clone of the original primary.

Clonality testing of this nature has been studied by numerous investigators over the past two decades. However, this period has been marked by rapid changes in genetic technology, and so the kinds of data available have evolved. Early studies typically involved examination of a few candidate markers for loss of heterozygosity (LOH), representing copy number changes in the genetic region of the marker locus [Imyanitov et al. (2002), Sieben et al. (2003), Dacic et al. (2005), Geurts et al. (2005), Orlow et al. (2009)]. The LOH profiles would then be compared to determine if the two tumors shared a clonal origin. Our group developed statistical tests designed for this comparison and applied these in studies of melanoma and breast cancer [Begg, Eng and Hummer (2007), Ostrovnaya, Seshan and Begg (2008)]. However, as the technology evolved, investigators were increasingly drawn to the use of genome-wide techniques for this purpose [Bollet et al. (2008), Girard et al. (2009)]. We have also examined in detail this framework and have developed methods for comparing the genome-wide copy number profiles for the purpose of clonality testing [Ostrovnaya et al. (2010), Ostrovnaya et al. (2011)]. The statistical framework for formulating the comparison of copy number profiles is radically different from the comparison of profiles of individual markers of LOH even though the fundamental goal of testing for clonal origin is exactly the same. The current era is marked by a further significant change in technology, the introduction of deep genetic sequencing [De Mattos-Arruda et al. (2014)]. This approach identifies individual somatic mutations within genes such as single nucleotide variants, deletions, insertions and other extremely localized events. These mutations are usually identified by comparing the tumor sample with a matched normal sample to screen out germ-line variants. Addressing the problem of clonality testing from sequence data is very distinct from the challenges presented in our earlier work on LOH and copy number profiles. In the former setting [Begg, Eng and Hummer (2007), Ostrovnaya, Seshan and Begg (2008)] we dealt with data on a limited number of markers where the marginal probabilities of allelic losses could reasonably be considered to be constant, greatly simplifying the construction of the test. Our work on copy number profiles was challenged by the problems of determining the locations of the allelic changes and then formulating a probabilistic strategy for determining if the locations of the changes could reasonably be considered to be identical [Ostrovnaya et al. (2010, 2011)]. With deep sequencing data, the major challenges are different (and imprecisely known) marginal probabilities of mutations at individual loci and the fact that the sample space of potential mutations is vast and can be defined only loosely.

Information on point mutations has gradually become more common in the clinic in recent years as specific driver genes have been identified, some of which have important therapeutic implications in that mutations in these genes may be targets for available drugs that have efficacy against tumors with these mutations. For example, mutations in the gene *EGFR* can be targeted by the drug erlotinib, while vemurafenib is especially effective against tumors with mutations in the gene

BRAF [Köhler and Schuler (2013), Jang and Atkins (2014)]. Consequently, such mutational data are likely to become increasingly available routinely to pathologists when diagnosing a new tumor in a patient with an existing tumor. It is our impression that pathologists will typically conclude that the tumors are clonal if they match on a single mutation of this nature. One of the goals we seek to address in this article is to answer the question: is this conclusion justified? More generally, we develop a framework for assessing the evidence for clonal relatedness of two tumors when there may be one or more mutations observed in each tumor, some matching and some nonmatching.

2. Motivating example and methods. We introduce the problem in the context of an interesting recent example published by Kunze et al. (2014). The data, displayed in Table 1, came from a patient with two primary colon cancers, denoted T1 and T3, and tumors in both the right and left lungs. Areas of the left lung tumor with distinct histological features were examined separately. The investigators were interested in whether or not the lung tumors could be metastases of one or other of the colon primaries. Since mutations in the gene *KRAS* are common in colon tumors the investigators performed *KRAS* mutation testing. They discovered distinct *KRAS* mutations in the left and right lung specimens, suggesting these tumors are independent, but noticed that the left lung tumor shared a *KRAS* G12D mutation with one of the colon primaries, suggesting that the left lung tumor might be a metastasis of the T3 colon primary. We know from much previous experience that *KRAS* G12D mutations occur in about 8% of colon cancers. Based on this fact, how strong is the evidence for a clonal link between these two tumors? Clearly a match is evidence in favor of clonal relatedness, but *KRAS* G12D is a common mutation and so it is not unlikely that two tumors might share this mutation simply by chance. A match at a location that is more uncommon would provide stronger evidence for clonality. To enhance the evidence the investigators elected to perform additional targeted next generation sequencing on a much more extensive panel of genes, and the remaining mutations detected are also displayed in Table 1. Here we see that the colon T3 primary has five additional mutations detected while the left lung tumor has 4 or 6 additional mutations depending on the histological region examined. However none of these additional mutations match the new mutations in the colon primary. Clearly, the presence of new nonmatching mutations diminishes the evidence favoring a clonal relationship, but how do we quantify the negative evidence in these nonmatches with the positive evidence in the *KRAS* G12D match?

2.1. *Assumptions and notation.* The key technical features of the problem are as follows. First, mutations could occur at a very large number of genetic loci, depending on the size of the sequencing panel used. We denote this number by n. We denote by m the number of loci at which mutations are actually observed in either tumor in the case under consideration. The marginal mutation probabilities at each locus, defined as p_i for the probability of a mutation at the ith locus, will

TABLE 1
Data From Kunze et al. (2014)

Mutation	Probability[1]	Colon tumors		Lung tumors		
		T1	T3	Right	Left/Tubular	Left/Mucinous
KRAS G12D	0.081		✓		✓	✓
KRAS G12S	0.019			✓		
XPA G74V	0.004		✓			
PIK3CA Q546P	0.004		✓			
FBXW7 R465C	0.004		✓			
APC R283*	0.004		✓			
APC R499*	0.004		✓			
APC Q1065*	0.004	✓				
TP53 R158H	0.004	✓				
BRAF G596V	0.004			✓		
BAI3 V499L	0.004			✓		
PIC3C2B S314F	0.004			✓		
ETS1 K200N	0.004			✓		
IKZF1 M301I	0.004				✓	✓
PRKDC R364H	0.004				✓	✓
ZNF521 L1136V	0.004				✓	✓
ALK E405*	0.004				✓	✓
GUCY1A2 V627A	0.004				✓	
ACVR2A A62G	0.004				✓	

[1]Mutations that were not observed in TCGA were assigned a marginal probability of $(a + 1)^{-1}$, where a is the number of cases observed in TCGA.

generally only be known approximately for the common hotspot mutations that have been frequently observed in the past, and must be small for mutations that have either never been observed or not previously observed prior to occurrence in the case under consideration. Since a match at a rare mutation is much less likely to occur by chance the observance of such a match provides greater evidence for a clonal origin for the tumors than a match at a common locus, so our testing procedure must recognize this. We have elected to use data from the National Cancer Institute-sponsored Cancer Genome Atlas (TCGA) to estimate these probabilities in our examples [Kandoth et al. (2013)]. Specifically, we aggregated frequencies observed in the TCGA database with data from the study in question to obtain an estimate. New solitary mutations that were not observed in TCGA were assigned a marginal probability of $(a + b)^{-1}$ where a is the number of TCGA cases from the cancer site under investigation and b is the number of cases in the study. In our testing procedure we assume that these marginal mutation probabilities are known exactly. Later we investigate the consequences of inaccuracies in these estimates. An additional key assumption in the statistical test in Section 2.2 below is indepen-

dence of mutations in different markers. This is not true for genes that are linked by some known genetic pathways [Sweeney et al. (2009)] and we also explore the implications of this simplifying assumption later in Section 3.3.

Finally, we assume that matching clonal mutations occur in the original clonal cell, but that at some point a cell from this clone travels to another site in the body and seeds the development of the metastasis. After this, the two tumors can continue to evolve independently through further mutation and the development of new dominant clones that contain both the original set of mutations and additional independent mutations. Thus clonal tumor pairs will possess identical mutations that occurred during the initial "clonal" phase of development and additional sets of distinct mutations in each tumor, most of which will be nonmatching but some of which could be identical by chance. To model this process we use a parameter, ξ, that characterizes the probability that a mutation will occur in the clonal phase as opposed to the independent phase. Thus $\xi = 0$ for independent tumor pairs and $\xi > 0$ represents the strength of the clonality signal. Clearly this is likely to vary from case to case. If ξ is large then clonal tumors will typically have very similar profiles, while if ξ is small then independently occurring mutations will predominate.

2.2. *A test for clonal relatedness.* Let n be the total number of distinct mutations (markers) that potentially could occur. Let A denote the set of markers at which a matching mutation occurs on both tumors, let B denote the set of markers at which a mutation occurs on one tumor but not the other, and let C denote the set of markers at which no mutations are observed. Further let D denote the set of all loci, that is, $D = A \cup B \cup C$, and let E denote the set of markers that experience mutations, that is, $E = A \cup B$. Applying the Neyman–Pearson lemma the most powerful test statistic for distinguishing clonal versus independent tumor pairs is of the form

$$S_u = \sum_{i \in A} \log\left[\frac{\hat{\xi}}{1 - \hat{\xi}} p_i^{-1} + 1 \right] - \sum_{i \in E} \log\left[\frac{\hat{\xi}}{1 - \hat{\xi}} (1 - p_i)^{-1} + 1 \right]$$

$$+ \sum_{i \in D} \log\left[\frac{\hat{\xi}}{1 - \hat{\xi}} (1 - p_i)^{-1} + 1 - \hat{\xi} \right].$$

The last term is summed over all markers. Hence is a constant, and the test reduces to weighted contributions from sets A and B, the markers at which mutations are actually observed.

For the generalized likelihood ratio test we use the maximum likelihood estimate of ξ in the test statistic. However, construction of a null reference distribution presents a major challenge. This depends on the distribution of the statistic generated from the full set of n markers. In practice markers with mutations will represent only a tiny fraction of n, which itself may be an extraordinarily large number. Consequently it is very appealing to approach the problem by using a

conditional test, conditioned on the set of markers with mutations observed in one or both tumors, that is, $i \in E$. In this conditional setting the likelihood ratio can be expressed as:

$$(1) \qquad S_c = \sum_{i \in A} \log\left[\frac{\hat{\xi}}{1 - \hat{\xi}} p_i^{-1} + 1\right] - \sum_{i \in E} \log\left[\frac{\hat{\xi}}{1 - \hat{\xi}} (2 - p_i)^{-1} + 1\right].$$

The last term is a constant since it is summed over all of the markers in the reference set for the conditional test. Consequently the conditional likelihood ratio test statistic depends solely on the weighted contributions of the markers with matched mutations on both tumors, weighted by $\log[(\hat{\xi} p_1^{-1}/(1 - \hat{\xi})) + 1]$, the same weights used for the matches in the unconditional test. The maximum likelihood estimate of ξ in the conditional setting can be obtained by maximizing the likelihood numerically under the constraint that $0 \le \hat{\xi} \le 1$, where, by definition, $\hat{\xi} = 0$ if no matches are observed and $\hat{\xi} = 1$ if all observed mutations are matched on both tumors.

The crucial practical advantage of the conditional test is that we can generate relatively easily a null reference distribution since the sampling depends solely on the markers with mutations observed in one or other of the patient's tumors. This number is relatively small in our examples, but it is likely to be manageable, at most in the hundreds, even if full genome sequencing is used, based on projections from the TCGA project. To obtain a reference distribution we generate the distribution of S_c under the assumptions that matches occur randomly through independent sampling and that at least one mutation is observed at each marker in the set E. Specifically, let q_i be the probability that there is a matching mutation at the ith marker given that the marker is mutated in at least one of the two tumors. Then under the null hypothesis that the tumors are independent

$$q_i = p_i^2/(p_i^2 + 2p_i(1 - p_i)).$$

To simulate the null distribution of S_c we randomly generate the matches over the set E, that is, for the ith marker of the jth of T simulations we generate x_{ij} as a Bernoulli random variable with probability q_i where $x_{ij} = 1$ if there is a match and 0 otherwise Then the test statistic for the jth simulation is given by

$$S_j = \sum_{i \in E}\left[x_{ij}\left\{\log\left(\frac{\tilde{\xi}_j}{1 - \tilde{\xi}_j} p_i^{-1} + 1\right)\right\} - \left\{\log\left[\frac{\tilde{\xi}_j}{1 - \tilde{\xi}_j}(2 - p_i)^{-1} + 1\right]\right\}\right]$$

$$\text{for } j = 1, \ldots, T,$$

where ξ_j is the MLE based on data from the jth simulation. The p-value is given by $\sum_{j=1}^{T} I(S_j > S_c)/T$. The critical value of this test at the one-sided α level, denoted k_α is the smallest value of k_α such that $\sum_{j=1}^{T} I(S_j > k_\alpha)/T < \alpha$.

3. Statistical properties. In the following we use simulations to address several questions about the preceding testing strategy. First, since the actual mutational profiles of tumors arise through a random process represented by unconditional sampling, is the conditional test valid? Further, in discarding information about the markers that were tested but exhibited no mutations in either tumor are we materially reducing the efficiency of the test? Second, given that in practice we must use estimates of the marginal mutation probabilities of the mutations that are observed in any tumor pair under consideration, how sensitive is the test to inaccuracies in these marginal probabilities? Third, to what extent are the properties of the test affected by correlations among mutations?

3.1. *Validity and efficiency of conditioning on observed mutations.* Because of the computational barriers to use the unconditional test when there are large numbers of markers that could harbor a mutation, allied to the fact that the unconditional test represents the gold standard against which to compare the conditional test, we have constructed simulations in configurations where the number of mutations observed reflect settings that we believe will be realistic, but where the total number of markers is chosen to be small enough to facilitate the comprehensive computation required in the unconditional setting. Thus we construct simulations in which $n = 10{,}000$. We calculate the size and power of the test for various combinations of the clonality signal, ξ, the number of markers with mutations observed in either or both tumors (m) and their associated marginal probabilities $\{p_i\}$.

In Table 2 we present the test characteristics in the setting in which the assumptions are correct, that is, the mutations are generated independently and the marginal mutation probabilities used in the test are accurate. The configurations of marginal probabilities reflect in general terms the nature of somatic mutation, namely, a few "common" markers with marginal probabilities of 0.1, and a large number of "rare" markers. We generated configurations in which the mean numbers of mutations observed per tumor are five, 10 and 20, with clonality signals of 0 (null), 0.1 and 0.25. Details of the actual marginal probabilities are provided in the table footnotes. The results show that the conditional test is valid. This can be seen in the column "Conditional Test" for null ($\xi = 0$) settings, that is, the size of the test is consistently less than the nominal 5% level of the test, regardless of the configuration of marginal probabilities. To compare the power of the conditional and unconditional tests we "calibrated" the results by randomization to ensure that the test size is always exactly 0.05. The results show that the conditional test has slightly lower power than the unconditional test, but that the bulk of the information appears to be captured by the conditioning.

3.2. *Inaccuracies in marginal probability estimates.* Our test depends on specification of the marginal mutation probabilities for each locus at which a mutation is observed. At this stage of genomic knowledge we do not have accurate

TABLE 2
Validity and efficiency of conditional test

Mean # mutations per tumor	Clonality signal	Mean # matching mutations	Frequency of $p < 0.05$[1]		
			Unconditional test (calibrated)	Conditional test	Conditional test (calibrated)
5	0.0	0.10	0.05	0.01	0.05
5	0.1	0.56	0.41	0.36	0.40
5	0.25	1.32	0.72	0.65	0.70
10	0.0	0.21	0.05	0.02	0.05
10	0.1	1.17	0.60	0.57	0.59
10	0.25	2.60	0.92	0.89	0.90
20	0.0	0.45	0.04	0.03	0.05
20	0.1	2.41	0.83	0.81	0.82
20	0.25	5.28	0.99	0.99	0.99

[1]Each row of the table involved a simulation with 10,000 markers in which the reference distribution for the test involved sampling from the null distribution 5000 times, and in which the test was repeated 1000 times to estimate the size (when the clonality signal is 0) or power (when the clonality signal is >0). The marginal frequencies were constructed in the following way: For configurations with five mutations per tumor, 10 of the loci had a marginal probability of 0.10 and the remaining 9990 had a marginal probability of 0.0004. For configurations with 10 mutations per tumor, 20 of the loci had a marginal probability of 0.10 and the remaining 9980 had a marginal probability of 0.0008. For configurations with 20 mutations per tumor, 20 of the loci had a marginal probability of 0.10 and the remaining 9960 had a marginal probability of 0.00016.

information for ths purpose so our strategy is necessarily ad hoc. We have addressed the likely consequences of misspecification from two perspectives. First, we generated simulations in which we added noise to the marginal probabilities used in the data analyses. This was accomplished by perturbing the "true" marginal probabilities $\{p_i\}$, that is, those used to generate the data, to $\{p_i^*\}$, using $\log\{p_i^*/(1 - p_i^*)\} = \log\{p_i/(1 - p_i)\} + \varepsilon_i$, where ε_i is a random $N(0, 0.5)$ error term. These incorrect frequencies were used both for calculating the test statistic and for generating the reference distribution. These errors correspond approximately to the statistical uncertainty in mutation probability estimates obtained for marginal probabilities of "common" markers in the range 0.05–0.10 from sample sizes in the range of 500–1000, roughly the current state of knowledge based on data from The Cancer Genome Atlas project [Kandoth et al. (2013)]. The results are in the column "Random Errors" in Table 3 and these can be contrasted with the results based on the true probabilities in the preceding column. The results demonstrate a very modest anti-conservative trend. A possibly greater concern is the fact that for the vast majority of potential mutational locations in the genome no previous mutation has been observed. Consequently each time a first occurrence is observed we have elected in practice to use a marginal estimator, N^{-1}, where N is the total number of patients examined to date, including those from

TABLE 3
Sensitivity of the test to inaccuracies in the marginal mutation probabilities

Mean # mutations per tumor[1]	Clonality signal	Mean # matching mutations	Frequency of < 0.05		
			Test calculated using		
			True probabilities[2]	Random errors[3]	Rare mutation overestimation[4]
5	0.0	0.10	0.01	0.02	0.01
5	0.1	0.56	0.36	0.37	0.33
5	0.25	1.32	0.65	0.67	0.65
10	0.0	0.21	0.02	0.04	0.00
10	0.1	1.17	0.57	0.58	0.37
10	0.25	2.60	0.89	0.90	0.80
20	0.0	0.45	0.03	0.06	0.00
20	0.1	2.41	0.81	0.82	0.54
20	0.25	5.28	0.99	0.99	0.94

[1]In all configurations the data are generated using the same set-ups as described in the footnotes to Table 1 with regard to the marginal probabilities of the mutations and the clonality signal.

[2]Here the test is computed by using the same marginal probabilities as were used in the data generation.

[3]For the purposes of calculating the test statistic and its reference distribution, the marginal probabilities of the markers $\{p_i\}$ were perturbed with random errors to $\{p_i^*\}$, using $\log\{p_i^*/(1 - p_i^*)\} = \log\{p_i/(1 - p_i)\} + \varepsilon_i$ where ε_i is a random $N(0, 0.5)$ error term.

[4]For the purposes of calculating the test statistic and its reference distribution, the marginal probabilities of the common markers are assumed to be correct but the probabilities of the rare markers are overestimated by a factor of 10.

publicly available databases like TCGA. It is highly probable that this will typically be an overestimate because of the large number of potential mutations in the genome and the fact that most of the mutations observed to date have only been seen in a single patient. To address the impact of this, we constructed simulations in which the marginal probabilities of all of the "rare" mutations used in the test were overestimated by an order of magnitude compared to the probabilities used in generating the data. These are displayed in the column headed "Rare Mutation Overestimation" in Table 3. This phenomenon makes the test more conservative since overestimation of the marginal probability reduces the strength of evidence favoring clonality, but clearly does not threaten test validity. However, substantial power is still apparent for the kinds of configurations examined.

3.3. *Impact of correlations between markers.* We address the influence of negative and positive correlation between markers separately. It is well known that genes that operate in carcinogenic pathways will often not be co-mutated in tumors. That is, a mutation in one gene in the pathway will be sufficient to lead to the tumorigenic effects needed. An example is the mutual exclusivity of *BRAF* and

NRAS mutations in melanomas. As a result, we know that strong negative correlations between mutations in different genes can occur. To model this kind of phenomenon we generate data where subsets of the markers are classified into groups of "pathways" such that co-incident mutations within a pathway are not possible, that is, there is exclusivity between mutations in each pathway. To accomplish this we simply generated a single outcome from a multinomial comprising all the markers in the pathway with an additional cell of the multinomial representing no mutation. Further details are in footnote 3 of Table 4 with results presented in the column "Negative Correlations." These results show that this phenomenon has little impact on either the size or the power of the test.

From an intuitive standpoint, positive correlation is a much more problematic phenomenon in principle since it will lead to a greater chance of pairs of matched loci occurring together, with the potential to greatly inflate the evidence favoring clonality when the reference distribution assumes independence of markers. To address this we generated data where once again the markers were grouped into "pathways." However, in contrast to the mutually exclusive set-up above, here sets of markers with pairwise positive correlations are generated within the pathways with the same marginal frequencies as for the corresponding "uncorrelated" framework. Briefly, markers within groups are generated as multivariate normal correlated variables which are then dichotomized to produce the desired marginal frequencies (further details are provided in the footnotes to Table 4). When the within-blocks correlation is a moderate 0.3 the impact on the test appears to be a modest reduction in power, but the power decreases notably when the correlations are high (0.9). The factors bearing upon this loss of power are complex. The increased tendency for joint occurrence of clonal pairs of correlated markers has an anti-conservative influence on the test, but this is offset by the diminished effective sample size due to the presence of correlation. Further discussion of this issue is provided in a supplementary material [Ostrovnaya, Seshan and Begg (2015)]. We believe that the scenarios used in Table 4 that suggest the typical overall effect will be conservative provide a persuasive picture of the likely impact in practice. To gauge this we examined the empirical correlations between common genes using the TCGA data for the four major solid tumors: breast, colon, lung, prostate. That is, we correlated genes rather than individual mutations: logically the latter correlations should be lower. We only looked at common genes since for rare mutations the vast preponderance of mutation pairs have never been observed to occur together, making it difficult to determine reliably the likely correlation structure. We cross-tabulated the occurrence of mutations in all pairs of genes that occur with greater than 10% frequency and derived the underlying distribution of pairwise correlations on the assumption that mutations are binary classifications from latent correlated normal variates. The 75% percentile of the pairwise tetrachoric correlations, computed using the "polychor" function from the R package "*polychor*," is 0.19 for prostate, 0.32 for lung, 0.15 for breast and 0.50 for colorectal, while the maximum values are 0.38 for prostate, 0.59 for lung, 0.35 for breast and 0.86

TABLE 4
Sensitivity of the test to correlations in the markers

| Mean # mutations per tumor[1] | Clonality signal | Mean # matching mutations | Frequency of < 0.05[1] | | Positive correlations[4] | |
			Uncorrelated[2]	Negative correlations[3]	0.3	0.9
5	0.0	0.10	0.01	0.04	0.02	0.02
5	0.1	0.56	0.34	0.41	0.31	0.24
5	0.25	1.32	0.64	0.66	0.62	0.46
10	0.0	0.21	0.02	0.02	0.02	0.03
10	0.1	1.17	0.57	0.55	0.56	0.40
10	0.25	2.60	0.87	0.87	0.84	0.67
20	0.0	0.45	0.04	0.02	0.04	0.05
20	0.1	2.41	0.81	0.83	0.75	0.55
20	0.25	5.28	0.98	0.99	0.98	0.87

[1] In all configurations, the 10,000 markers are generated using the same marginal probability set-ups as described in the footnotes to Table 2 with regard to the marginal probabilities of the mutations and the clonality signal.

[2] Here the test is computed by using the same marginal probabilities and (uncorrelated) data generation as in Tables 2 and 3.

[3] In these configurations, negative correlation between "pathways" is generated as follows, designed such that the overall mean numbers of matching mutations are equivalent to the corresponding uncorrelated configuration. Common markers are generated in blocks of 10 using a single draw from a multinomial distribution in each block with 10 mutually exclusive outcomes and fixed marginal frequency of 0.1 each. One, two or four such multinomials, respectively, are generated for each tumor under the three scenarios (mean # mutations of five, 10 or 20). In addition, 5000 rare markers are generated in 50 blocks of mutually exclusive markers of size 100 and fixed rare marginal frequencies for each mutation (4/9990, 8/9980 and 16/9960, resp., for the three scenarios). That is, we generated one draw from each multinomial with 101 potential outcomes where none of the 100 markers exhibit a mutation when 101th outcome is selected (probability of the 101st outcome is 9490/9990, 9180/9980 and 8360/9960, resp., for the three scenarios). For markers that belong to these multinomial blocks, the clonality status is drawn once for a whole block, that is, the whole blocks rather than individual mutations are considered clonal or independent. The remaining 4990, 4980, or 4960 markers in the three scenarios are independent of each other and of multinomial blocks and are generated as described above. The test statistic and reference distribution are calculated assuming all markers are independent.

[4] Similar to (3) above, one, two or four blocks of size 10 of common markers and 50 blocks of size 100 of rare markers are generated with positive correlation. To accomplish this we generated multivariate normal variates Y of size 10 or 100 with 0 mean, variance 1 and pairwise correlations of 0.3 or 0.9. The correlated binary mutation outcomes were determined by dichotomizing these normal variables at the appropriate marginal frequencies. Clonality status was drawn on per-block basis as described in (3) above, and the remaining markers were generated independently. Note that in this set-up it is possible for greater than one mutation to be observed within a block (indeed this is increasingly likely as the correlation increases) while in the mutually exclusive construct in (3) above at most one mutation is observed in each block.

for colorectal. That is, very high correlations will only occur occasionally and the preponderance of the gene pairs have correlations that are sufficiently low to have at worst very modest impact on the power of the test.

4. Examples. We illustrate the test using examples from the recent literature. Both studies involved mutational profiling addressing the clonality of primary metastasis pairs of tumor specimens, as described earlier in Section 2. In the example from Kunze et al. (2014) the investigators performed next generation sequencing on two colorectal tumors considered clinically to be multifocal and two nonsmall cell lung cancers, one in the left and one in the right lung, all identified synchronously in the same patient (Table 1). Recall that initially the investigators had only information regarding a matching mutation between the T3 colon tumor and the tumor in the left lung at *KRAS* G12D. Our test based on this single locus has a *p*-value of 0.042. Additional mutational testing identified five new mutations in the T3 lesion and either four or six additional mutations in the mucinous and tubular portions of the left lung lesion, respectively, though none of these were matched in the colon versus lung comparisons. By accounting for these additional nonmatches, our test leads to *p*-values of 0.063 and 0.067 when comparing the colon T3 with the mucinous and tubular lung lesions, respectively. This shows that nonmatches contribute small amounts of evidence against clonality and that this negative evidence will accumulate as more nonmatches are observed.

We have also analyzed another published example of a celebrated recent study involving panel sequencing of nine distinct local foci of prostate cancer, a lymph node metastasis and a series of distant metastases obtained from autopsy specimens many years after the primaries were surgically removed [Haffner et al. (2013)]. Mutations were identified in the *PTEN*, *TP53*, *SPOP* and *ATRX* genes. The data in Table 5 show that the four metastases are all clearly related through matches in at least three of these four genes ($p < 0.001$ for tests of any pair of metastases). Six of the primary specimens have no matches with the metastases. One primary, denoted P1, matches with the metastases on the *PTEN*, *TP53*, and *SPOP* mutations ($p < 0.001$) and the authors concluded that this tumor, the least advanced of the nine primary specimens on a histopathologic basis, contains the lethal clone that led to the metastases. However, two of the other primaries have matches with the metastases on the mutation *SPOP* F133L, and so it is pertinent to address the statistical evidence that these primaries may be clonally related to each other and to the metastases. In fact, a comparison of either P6 or P8 with P1 is significant ($p = 0.02$), and a comparison of either P6 or P8 with any of the metastases is also significant ($p = 0.02$). In short, the authors' interpretation that the low grade P1 tumor is the sole primary tumor that is related to the metastases may be an incomplete explanation of the clonal evolution of these tumors. Interestingly, the local lymph node possesses none of these mutations and it is entirely possible that it is linked clonally to some of the other primary tumors through shared mutations in genes that were not tested. More extensive mutation testing of additional genes would be needed to fully resolve the clonal development of these tumors.

TABLE 5
Data from Haffner et al. (2013)

Site	Tumor	Observed mutations			
		PTEN del.	TP53 R248Q	SPOP F133L	ATRX inversion
Mutation probabilities →		0.004	0.008	0.023	0.004
Prostate	P1	✓	✓	✓	
	P2				
	P3				
	P4				
	P5				
	P6			✓	
	P7				
	P8			✓	
	P9				
Local Node	L1				
Lung	B1	✓	✓	✓	
Liver	M5	✓	✓	✓	✓
Gastric Node	M38	✓	✓	✓	✓
Lung	M40	✓	✓	✓	✓

Mutations that were not observed in TCGA were assigned a marginal probability of $(a+1)^{-1}$, where a is the number of cases observed in TCGA.

We note that in both these examples several tests were performed between different tumor pairs from the same patient in order to explore the possible relationships between individual tumor pairs. We have not performed any multiple testing adjustments. We note that these tests are structurally dependent, and indeed the deciphering of the full set of clonal relationships among multiple tumors in a single case represents a more complex problem that is beyond the scope of this article.

5. Discussion. Clonality testing using sequencing is likely to be an emerging clinical application. It has been known for at least two decades that more accurate pathological diagnosis of metastases is possible using mutational testing of tumor samples. However, as yet, routine mutational testing has not entered the clinic. We are now entering an era in which routine clinical testing of tumor samples is likely to become commonplace as oncologists seek to identify actionable mutations for targeted therapy. In the medium-term the technologies will involve deep sequencing of panels of genes likely to harbor mutations of therapeutic potential, such as the one employed in Wagle et al. (2012). A by-product of such testing will be the availability of mutational data to test the clonal relatedness of metastases with their putative primaries. We have provided a testing strategy to perform such classifications, one that is conceptually and computationally straightforward to apply, and which appears to enjoy good statistical properties.

An unusual conceptual challenge in constructing a test in this context if the fact that the sample space is ill-defined due to the fact that it is not possible to specify precisely the number of potential ways in which a DNA mutation can occur. Additionally, for most realistic gene panels, the number of potential mutations is extremely large, making it computationally challenging to establish a reference distribution for any test statistic. We circumvented these problems by constructing a conditional test, conditional on the actual set of mutations observed. We showed through simulation that this conditional test is valid and captures most of the relevant information.

Our examples showed that strong evidence for clonal relatedness is possible even if matches are observed in only two genes. However, a match in a single, recurring mutation in major cancer genes such as *KRAS*, *BRAF*, *PTEN*, etc., may not provide sufficient evidence to demonstrate clonal relatedness convincingly. Stronger evidence is possible if the match occurs at a more rarely occurring genetic locus. If clonality testing were to be performed routinely in the clinic the gene panel should ideally be sufficiently large to ensure that several mutations will be observed in all cases encountered.

A notable limitation to our approach is the fact that we must assign values to the marginal probabilities at each locus. In our example we used empirical relative frequencies as estimates for the marginal probabilities, derived using the publicly available TCGA data. This raises the question of how to update these marginal estimates, and in particular how to assign probabilities for mutations seen for the first time in a new patient, something that is likely to occur quite frequently. If one uses smaller marginal probability estimates for nonrecurring mutations based on the recognition that there are huge numbers of genetic loci at which mutations can potentially occur then the p-values will be smaller. We advocate an estimation strategy that results in the test being somewhat conservative, as shown in the simulations. Our simulations of settings in which we substantially overestimated the marginal probabilities demonstrated the degree of conservativeness to be expected if these probabilities are overestimated by an order of magnitude. As evidence gradually accrues about the frequency of specific mutational events in cancers, our uncertainty about the assignment of marginal probabilities will decrease, notably for the more commonly occurring mutations. Our test is also based on the assumption that mutational events occur independently at different loci. While this clearly is not literally true across the genome, our investigation of the impact of departures from independence showed that the kinds of dependencies observed between mutations in the TCGA project are likely to have modest impact on the properties of the test.

As a final cautionary note it must be recognized that all mutations from sequencing panels are called after a complex laboratory and data normalization process that can be influenced by numerous potential biases, including contamination of the specimen with normal cells and various laboratory processing artifacts. Also, tumors are heterogeneous and some mutations may only be present in a small subset

of tumor cells and thus detectable only if sequencing coverage is sufficiently high. These artifacts can introduce false positives or false negatives. In short, reaching a definitive diagnosis of clonal relatedness simply because one of many observed mutations is determined to be matched in the two tumors may overstate the true strength of the evidence, even if the matching locus is a "rare" locus. However, with proper curation of the called mutations false positive matches due to germline effects or other artifacts are unlikely.

SUPPLEMENTARY MATERIAL

Supplementary appendix (DOI: 10.1214/15-AOAS836SUPP; .pdf). Explanation of results of simulations with correlated markers. In the on-line supplementary appendix we provide additional calculations and graphs that provide an explanation of the power trends in the presence of correlated markers..

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