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Testing clonality of three and more tumors using their loss of heterozygosity profiles

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Abstract

Cancer patients often develop multiple malignancies that may be either metastatic spread of a previous cancer (clonal tumors) or new primary cancers (independent tumors). If diagnosis cannot be easily made on the basis of the pathology review, the patterns of somatic mutations in the tumors can be compared. Previously we have developed statistical methods for testing clonality of two tumors using their loss of heterozygosity (LOH) profiles at several candidate markers. These methods can be applied to all possible pairs of tumors when multiple tumors are analyzed, but this strategy can lead to inconsistent results and loss of statistical power. In this work we will extend clonality tests to three and more malignancies from the same patient. A non-parametric test can be performed using any possible subset of tumors, with the subsequent adjustment for multiple testing. A parametric likelihood model is developed for 3 or 4 tumors, and it can be used to estimate the phylogenetic tree of tumors. The proposed tests are more powerful than combination of all possible pairwise tests.

Keywords

clonality; LOH; metastasis; multiple tumors; concordant mutations test; likelihood ratio test

1. Introduction

Studying tumor loss of heterozygosity (LOH) profiles can help establish whether tumors from the same patient have developed independently or are clonal, i.e. originated from the same cancerous cell. Such a diagnosis has important clinical implications for the patient, and it is needed in various clinical scenarios when the distinction cannot be made by the pathologists. The data for such tests consist of binary mutational status at each of the a priori selected genetic markers of every tumor, with additional information on whether the LOH at the particular marker has happened on the same or opposite alleles. Since LOH in different alleles is not comparable across markers, the outcome at each marker cannot be described by 3 separate categories, and therefore methods developed, for example, for measuring inter-observer agreement do not apply in this setting. Usually loci with relatively frequent

Software:
The described methods are implemented in R/Bioconductor package Clonality (http://www.bioconductor.org/packages/devel/bioc/html/Clonality.html)

mutations are used, and often markers are selected from different chromosomes where mutations are independent. Previously we have successfully developed statistical methods for testing whether two tumors from the same patient are clonal or independent based on these LOH profiles (Begg et al., 2007; Ostrovnaya et al., 2008). In many studies, however, patients may present with 3 or more tumors; see, for example, Orlow et al., 2009; Lindgren et al., 2008; Hampl et al., 1999. Some studies specifically focus on triplicates of tumors where clonality of all of them is in question, e.g. ductal and lobular carcinoma in situ and invasive carcinoma (Wagner et al., 2009) or three lung tumors (Froio et al., 2008). Similarly, several lesions are often compared at once in multifocal cancers or in the case of multiple metastases (Wang et al., 2009; Lin et al., 2008; Irving et al., 2005; Bahrami et al., 2007; Jones et al., 2006; Khalique et al., 2009). Another similar application includes studies of tumor heterogeneity that use subsamples of the same tumor (Khalique et al., 2007).

Since methods for comparing two tumors are already available, it is reasonable to analyze such multiple tumors by testing clonality of all possible pairs. However, this strategy has some flaws. Multiple comparisons will inevitably lead to higher error rates, and sometimes multiple tests may show inconsistent results. For example, if 3 tumors are available, either only one pairwise comparison or all three of them must be clonal. The diagnosis is not obvious when only two of the pairwise tests are significant. Another issue to consider is statistical power. Concordant mutations in the same marker in all the tumors are very unlikely to occur by chance in the absence of clonality (unless the mutation frequency is close to 1, which is not the case for most markers), and pairwise comparisons fail to recognize strong evidence of this nature. Another reason to analyze multiple lesions simultaneously is that many investigators are interested in inferring the phylogenetic tree of tumors that will describe their evolutionary history.

We propose two frameworks for testing clonality of multiple tumors simultaneously that will overcome the disadvantages of pairwise testing. In Section 2 we develop a non-parametric test that builds upon the Concordant Mutations Test for comparing two tumors (Begg et al., 2007). The test statistic is the number of markers with concordant mutations in all tumors. Its reference distribution under the null hypothesis of independence is derived sequentially using the hypergeometric distribution, assuming an LOH event is equally likely to occur at each marker and at either allele. In Section 3 we will extend the parametric likelihood of (Ostrovnaya et al., 2008) to 3 and 4 tumors. Here we specify the multinomial likelihood of the data for a given tumor topology. It is a function of parameters that represent the shared mutational history between tumors. Frequencies of mutations at each marker are assumed to be known and can be different. This model, however, is difficult to extend to 5 or more tumors. We demonstrate the real application of these tests in Section 4 and investigate their operating characteristics using simulations in Section 5. The last section contains discussion.

2. Nonparametric framework - Extended Concordant Mutations test

2.1 Subset-specific test

Suppose we are interested in testing clonality of K tumors, where $K \geq 3$. The null hypothesis is that the tumors are independent, and the alternative hypothesis is that some tumors are

clonal. Let the total number of markers be *J*, and assume that the markers are independent. This is usually expected when the markers are located on different chromosome arms. The test statistic for the Concordant Mutations (CM) test, introduced in (Begg et al., 2007), is a number of markers with concordant LOH in both tumors, i.e. LOH on the same parental allele. The most obvious extension of this statistic to multiple tumors is a number of markers with concordant LOH in all K tumors, and we call this test the Extended Concordant Mutations (ECM) test. By conditioning on the marginal counts of LOH in the tumors, we can calculate the reference distribution under the null hypothesis. Like the original CM test, the ECM test relies on the two assumptions: that the probability of LOH is the same for each marker, and that maternal and paternal allele mutations are equally likely.

Let m_i be the total number of LOH events in tumor *i*, *i* =1, …, *K*. Without loss of generality we can assume $m_1 \geq \ldots \geq m_K$. Let e_{12} be the total number of markers with LOH in both tumors 1 and 2, let a_{12} of these losses be on the same parental allele, i.e. concordant LOH. Let e_{123} be the number LOH in tumors 1, 2 and 3 and let a_{123} be the number of these that are concordant. Similarly we define $e_{123..K}$ and $a_{123..K}$ based on all K tumors. The statistic for testing the hypothesis that the K tumors are clonal is $a_{123..K}$, and we compute its distribution under the null hypothesis assuming $m_1, \ldots, m_K$ are fixed. In this section we will concentrate on K =3 and K =4, but all the described results can be easily generalized for higher K.

As shown in (Begg et al., 2007) for tumors $P(e_{12}|m_1, m_2) = H(e_{12}, m_1, m_2, J)$, where

$$H(e_{12}, m_1, m_2, J) = \frac{\binom{m_2}{e_{12}}\binom{J-m_2}{m_1-e_{12}}}{\binom{J}{m_1}}$$

is a hypergeometric distribution. If we assume that the losses of maternal and paternal alleles are equally likely, then concordant LOH is as likely as discordant LOH under the null hypothesis, and

$P(a_{12}|e_{12}, m_1, m_2) = Bin(a_{12}, e_{12}, \frac{1}{2})$ is a binomial probability of a_{12} successes out of e_{12} trials with probability ½. Then,

$$P(a_{12}|m_1, m_2) = \sum_{i=a_{12}}^{m_2} P(a_{12}|e_{12}=i, m_1, m_2)\, P(e_{12}=i|m_1, m_2)$$

Using the distribution of e_{12} we can calculate distributions of higher order counts of concordant mutations:

$$P(e_{123}|m_1, m_2, m_3) = \sum_{e_{12}=e_{123}}^{m_2} P(e_{12}|m_1, m_2)\, H(e_{123}, e_{12}, m_3, J)$$

There are 8 possible permutations of maternal and paternal allele LOH in a triplicate and in only 2 of them are all 3 mutations of the same type, therefore

$P(a_{123}|e_{123}, m_1, m_2, m_3)=Bin(a_{123}, e_{123}, \frac{1}{4})$. Thus the distribution of a_{123} is

$$P(a_{123}|m_1, m_2, m_3)= \sum_{i=a_{123}}^{m_3} P(a_{123}|e_{123}=i, m_1, m_2, m_3)\, P(e_{123}=i|m_1, m_2, m_3)$$

Using the same logic we can continue to 4 tumors:

$$P(e_{1234}|m_1, \ldots, m_4)= \sum_{e_{123}=e_{1234}}^{m_3} P(e_{123}|m_1, m_2, m_3)\, H(e_{1234}, e_{123}, m_4, J),$$
$$P(a_{1234}|e_{1234}, m_1, \ldots, m_4)=Bin(a_{1234}, e_{1234}, \tfrac{1}{8})$$

$$P(a_{1234}|m_1, \ldots, m_4)= \sum_{i=a_{1234}}^{m_4} P(a_{1234}|e_{1234}=i, m_1, \ldots, m_4)\, P(e_{1234}=i|m_1, \ldots, m_4)$$

The p-value can be calculated as a tail probability of the reference distribution of the test statistic with continuity correction, i.e. half the probability of the observed value of the test statistic added to the sum of the probabilities of the values above it. Since the ECM test is available for any number of tumors, it can be performed in every possible subset of *K* tumors.

2.1.1 Power and type I error of subset specific ECM test—We evaluate power and type I error of the ECM test in a simple simulation study where all assumptions of the ECM test hold and the specific subset of tumors is tested. We simulated sets of 4 tumors with *J*=15 markers, with probability of LOH at each marker equal to p_i =0.3, *i* =1,…,*J*. Four scenarios were simulated wherein none, 2, 3 or all 4 tumors were clonal. For all subsets of clonal tumors we assume that on average half of the LOH events have happened in the clonal phase, i.e. before the tumor metastasized. This proportion is represented by parameter *c*=0.5 and represents strength of the clonality signal. Shared mutations were generated in the clonal phase with probability cp_i, and if clonal mutation had not occurred then subsequent independent mutations were generated with probability $\frac{(1-c)p_i}{1-cp_i}$. Each mutation event is randomly assigned to maternal or paternal allele with probability 0.5. The simulation was repeated 1000 times. There are 11 possible tests we can perform when K=4: 6 pairwise tests, 4 three-way tests and one 4-way test. The test that focuses on mutations in the subset of tumors $\{I\}$ is denoted as $ECM_{\{I\}}$. In Table 1 we compare performance of these tests. Some subsets that were equivalent to subsets shown were omitted.

All the tests have type I error around or below a nominal level of 5% when the null hypothesis is true, i.e. all tumors are independent. When some of the tumors are clonal, the ECM test that evaluates this clonal subset is the most powerful test as we would expect. For

example, if tumors 1 and 2 are clonal, ECM_{12} has 63% power, while higher order tests that include subset {1,2} have much lower power. In this case the null hypothesis tested by ECM_{123}, for example, is not true, since not all 3 tumors are independent, but it is rejected only in 18% of cases, so this test is not powerful against the alternative hypothesis that only 2 tumors are clonal. However, if tumors 1, 2 and 3 are clonal ECM_{123} has 90% power, much higher than either of the pairwise lower order tests that are included in {1,2,3}. When all 4 tumors are clonal, ECM_{1234} is marginally better than the lower order 3-way tests, and again much better than the 2-way tests. From this simulation study we conclude that the ECM test can be much more powerful than the pairwise CM tests, however, it is most powerful when the correct subset of tumors is tested.

2.2 Inferring clonal relationship of multiple tumors

We consider two potential goals of clonality testing. The first goal is to determine whether any set of the tumors is clonal; this case is of potential clinical importance when the treatment strategy might depend on the results, e.g. more aggressive treatment is indicated when some tumors are clonal. Another goal is to determine the particular subset of tumors that are clonal; this knowledge can be of relevance in determining patterns of spread of the cancer, or for investigating somatic mutations associated with metastasis. For example, (Wagner et al., 2009) consider patients with 3 lesions, invasive ductal carcinoma (IDC), lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS). Clonality of IDC and DCIS would suggest a very different pathway of disease development compared to the situation where only LCIS and DCIS were clonal.

2.2.1 Testing whether any set of tumors is clonal—Let us denote all possible subsets of {1,..,K} by S_j, j=1,…, $K_0=2^K - K - 1$. Let H_{0,S_j} be a null hypothesis that all tumors in the subset S_j are independent, and $H_{A,\ S_j}$ the alternative hypothesis that at least 2 tumors in the subset are clonal, and let q_i be the p-value for the corresponding ECM_{S_j} test. The goal is to test the global null hypothesis that all tumors are independent: $H_0=\cap_{\{j\}} H_{0,S_j}$. We suggest that the most powerful strategy is testing all possible subsets of tumors, with proper adjustment for multiple comparisons. We will formulate the testing procedure in terms of adjusted p-values. Let q_{r_j} be ordered p-values, $q_{r_1} \le q_{r_2} \le \cdots q_{r_{K_0}}$. The classical approach that allows one to control the probability of a false rejection of H_0 below α is the Bonferroni method, where the adjusted p-value is equal to $\tilde{q}_J^{\,B}=\frac{q_j}{K_0}$. If $q_J^{B} \le \alpha$ for any j, then H_0 is rejected. Note that even if H_0 is true, the ECM p-values corresponding to the overlapping subsets are dependent since, for example, the number of concordant mutations in 3 tumors can only be as high as the number of concordant mutations in any nested pair of tumors. The permutation-based minP method (Westfall and Young, 1993) is more powerful when tests are dependent. The step-down minP adjusted p-values are defined as

$$\widetilde{q_{r_J}}^{M}=\max_{n=1,\ldots j}\{Pr\left(\min_{n\le l\le K_0} Q_{r_l} \le q_{r_n} | H_0\right)\},$$

where r_l are the ranks of the p-values, and Q_l are the random variables with the same distribution as p-values q_l. If for any j $\widetilde{q_{(r_j)}}^M \leq \alpha$, then H_0 is rejected. To obtain the reference distribution for min$_{n\leq l\leq K₀}$ Q_{r_l} under H_0 we can use the following algorithm:

1. Generate sets of K tumors with the same marginal counts of LOH $m_1,\ldots,m_K$. This can be accomplished by randomly permuting m_i LOH events across markers in tumor i, $1 \leq i \leq K$, and assigning paternal or maternal affected allele status with probability 0.5.
2. For each simulated set of tumors, perform all possible ECM tests, then take the minimums of successive sets of $r_n,\ldots,r_{K₀}$ -th p-values, $n = 1,\ldots,K_0$.
3. Repeat steps 1)–2) 1000 times to estimate $Pr(\min_{n\leq l\leq K₀} Q_{r_l} \leq q_{r_n}|H_0)$.
4. Reject H_0 if $\widetilde{q_{(1)}}^M = Pr\left(\min_{1\leq l\leq K_0} Q_l \leq \min_{1\leq l\leq K_0} q_l | H_0\right) \leq \alpha$

2.2.2 Identifying clonal subset—If H_0 is rejected, it would be of interest to identify which subset or subsets of tumors are clonal. The subsets S_j for which the adjusted p-values are significant would be natural candidates. We propose to take the union of all such subsets if they contain the same tumors; for example, if tests corresponding to {12} and {23} are significant, we conclude that the set of clonal tumors is {123}, while if {12} and {34} are significant, we conclude that tumors 1 and 2 are clonal, both independent from tumors 3 and 4, which are also clonal.

The methods described in this subsection will be evaluated in simulations in Section 5.

3. Parametric framework – Likelihood model

The ECM test makes several important assumptions: that the probability of mutation is the same across markers, and that the probabilities of maternal or paternal allele mutations are equal. In reality these assumptions do not necessarily hold. Another limitation of the tests of clonality of specific subsets is that p-values cannot be used as distance metrics between tumors since they don't satisfy the triangle inequality. Thus, it is not easy to establish the phylogenetic tree of the tumors, and there is no natural way to rank the subsets of tumors according to length of their shared history. In order to overcome that we extended the likelihood framework, previously derived for 2 tumors in (Ostrovnaya et al., 2008) to 3 and 4 tumors.

We have introduced the following parameters in the model for 2 tumors:

- $p_1, \ldots, p_J$ are the frequencies of LOH at markers 1 to J, assumed to be known in advance. This is a reasonable assumption since the markers are usually selected based on previous studies that can be used to estimate the frequencies.
- Parameter π represents the potential imbalance in the probability of LOH with respect to the maternal and paternal alleles. We define π as the conditional probability that, given that LOH occurs, it occurs at the favored allele, $0.5 \leq \pi \leq 1$.

If $\pi > 0.5$, then concordant mutations are more likely to be observed even in independent tumors.

- The strength of the clonality signal is represented by the parameter c, $o \le c \le 1$, defined as the probability that, given that LOH occurs, it occurs in the clonal phase of the tumor development. If $c = 0$, tumors are independent, and if $c = 1$, the tumors have identical patterns of LOH. The intuition behind this parameter is that $c \times 100\%$ of mutations have occurred in the clonal phase, i.e. before the tumor cell colonies split up, and $(1 - c) \times 100\%$ of mutations occurred when tumor cells have already diverged and were developing independently.

At each marker there are the following mutually exclusive possible outcomes x_j at two tumors: x_1 = "*S*,*S*" if there is LOH at both tumors and it is concordant, x_2 = "*S*,*L*" if there is LOH at both tumors but it is discordant, x_3 = "0,*S*" if there is LOH at one tumor but not in the other, and x_4 = "0,0" if there is no LOH at either tumor. In these notations 0 represents no LOH, and *S* and *L* represent whether LOH affects maternal or paternal alleles – these cannot be distinguished and are interchangeable, they are only used to denote concordance or discordance of LOH. Thus, for example, observing "*S*,*S*" is equivalent to observing "L,L" and both of these are described by one outcome x_1. Let z_i be the observed LOH outcome in two tumors at marker *i*. It has multinomial distribution with 4 categories and $n = 1$. We can write down the probabilities of these categories, initially introduced in (Ostrovnaya et al., 2008), as $\mathrm{h_i}^{x_j} = P(z_i = x_j)$, j=1,..,4, where

$$\mathrm{h_i}^{\text{"S,S"}} = cp_i + \frac{(1-c)^2 p_i^2}{1-cp_i}(\pi^2 + (1-\pi)^2), \quad \mathrm{h_i}^{\text{"S,L"}} = \frac{(1-c)^2 p_i^2}{1-cp_i}(2\pi(1-\pi)),$$
$$\mathrm{h_i}^{\text{"0,S"}} = \frac{(1-c)p_i(1-p_i)}{1-cp_i}2, \qquad \mathrm{h_i}^{\text{"0,0"}} = \frac{(1-p_i)^2}{1-cp_i}.$$

The multinomial likelihood for tumors 1, 2 can be written as:

$$L_{1,2}(c,\pi) = \prod_{i=1}^{J} [\mathrm{h_i}^{\text{"S,S"}}]^{1_{z_i=\text{"}S,S\text{"}}} \times [\mathrm{h_i}^{\text{"S,L"}}]^{1_{z_i=\text{"}S,L\text{"}}} \times [\mathrm{h_i}^{\text{"0,S"}}]^{1_{z_i=\text{"}0,S\text{"}}} \times [\mathrm{h_i}^{\text{"0,0"}}]^{1_{z_i=\text{"}0,0\text{"}}}$$

The likelihood ratio (LR) is calculated as $\frac{L_{1,2}(\hat{c},\hat{\pi})}{L_{1,2}(0,\hat{\pi}_0)}$, where $(\hat{c}, \hat{\pi})$ is the MLE and $\hat{\pi_0}$ is the MLE under the hypothesis that c=0. The likelihood is maximized using numerical optimization. The distribution of LR can be estimated using tumors simulated under $c = 0$, known mutation frequencies p_i and $\pi = \hat{\pi_0}$.

3.1 Likelihood model for 3 tumors

When modeling the relationship of 3 tumors we need to allow for possibility that two tumors out of 3 might share a stronger clonality signal. Graphically the mutational history of 3 tumors can be illustrated by Figure 1. We assume that parameter c pertains to all 3 tumors, and we introduce another parameter, c_{12}: $c_{12} \ge 0$, $c + c_{12} \le 1$. This parameter represents the additional time that the tumors 1 and 2 spent as part of the same tumor colony. Intuitively, in

this scenario c ×1 00% of mutations have occurred in the clonal phase, and then tumor 3 has separated and continued to develop on its own, while tumors 1 and 2 separated only after they have accumulated $(c + c_{12}) \times 100\%$ common clonal mutations. If $c = 0$, $c_{12} > 0$, then only tumors 1 and 2 are clonal; if $c > 0$, $c_{12} = 0$, then three tumors share equal clonality signal. All tumors are independent if $c = c_{12} = 0$. Analogously, we can define two more topologies with tumors 1 and 3 sharing additional signal c_{13}, or tumors 2 and 3 sharing c_{23}, with the constraints that $c + c_{13} \le 1$, $c + c_{23} \le 1$, and at most one of c_{12}, c_{13}, $c_{23} \ge 0$ is positive

The probabilities of possible outcomes at each marker can be calculated in a way similar to 2 tumors. We denote event i in tumor 3 and events j and k in tumors 1 and 2 by "j, k: i". Here the semicolon indicates that tumors 1 and 2 potentially share stronger clonality signal c_{12}. Possible outcomes x_j are "S,S;S", "S,S;L", "L,S;S" and so on. The probabilities of all possible outcomes $\mathrm{h}_{\mathrm{i}}^{x_j} = \mathrm{P}(\mathrm{z}_i = x_j)$, j=1,…,10 are given in Appendix A1. The likelihood under the first topology is equal to $L_{1,2;3}(c, c_{12}, \pi) = \prod_{i=1}^{J} \prod_{j=1}^{10} [\mathrm{h}_{\mathrm{i}}^{x_j}]^{1_{z_i = x_j}}$. The likelihood models for two other topologies are defined similarly. In all simulations in this paper we keep π fixed and equal to 0.5 both while generating the data and in the likelihood computation, but the likelihood could potentially be maximized over π as well. The three tumors are not interchangeable, so even under the null hypothesis of independence ($c = c_{12} = c_{13} = c_{23} = 0$) likelihoods under different topologies $L_{1,2;3}(0,0, \pi)$, $L_{1,3;2}(0,0, \pi)$, $L_{2,3;1}(0,0, \pi)$ might be different. To test the global null hypothesis we define the test statistic as

$$LR_3 = \max\left(\frac{maxL_{1,2;3}(\text{“}\pi)}{L_{1,2;3}(0,0,\pi)}, \frac{maxL_{1,3;2}(\text{”}\pi)}{L_{1,3;2}(0,0,\pi)}, \frac{maxL_{2,3;1}(\text{“}\pi)}{L_{2,3;1}(0,0,\pi)}\right).$$

Although LR_3 is not strictly a likelihood ratio, we further refer to this method as LR method. The distribution of LR_3 is estimated using independent tumors simulated with given frequencies p_i. If the null hypothesis is rejected, the phylogenetic tree of tumors can be obtained by choosing the tree and order of tumors that have the highest likelihood ratio $\frac{maxL_{\cdot}(\text{”}\pi)}{L_{\cdot}(0,0,\pi)}$.

3.2 Likelihood model for 4 tumors

There are two possible topologies (Figure 2) that can describe 4 tumors, and neither of them can be obtained by changing the order of the tumors or reparametrization of another topology. A third parameter has to be introduced. Topology 1, which can be fitted to 3 possible orderings of the tumors, allows two pairs of tumors that are clonal within pairs but are independent between pairs, while topology 2, which can be fitted to 12 possible orderings, allows three tumors to be clonal among each other and independent of the 4$^{\text{th}}$. One particular ordering of tumors for each topology is shown in Figure 2. The null hypothesis of independence is equivalent to $c = c_{12} = c_{34} = 0$ for Topology 1 and $c = c_{234} = c_{34} = 0$ for Topology 2. There are some scenarios where these two topologies are equivalent, for example, when $c_{12} = 0$ and $c_{234} = 0$, or $c > 0$ and $c_{12} = c_{34} = c_{234} = 0$.

The number of possible LOH outcomes in one marker that can be seen in 4 tumors and 2 topologies increases dramatically. Their probabilities are shown in the supplementary information attached. As with 3 tumors, the likelihood-based test statistic is defined as a maximum of likelihood ratios fitted to both topologies and all possible tumor orderings, and its distribution under the null is estimated using simulations. Once the null hypothesis is rejected, we choose the topology and ordering of tumors that has the highest likelihood ratio. Since multinomial distribution has more outcomes under topology 2 than under topology 1, likelihood values cannot be compared directly for this purpose.

It is possible to extend this likelihood model to 5 and more tumors; however, the analytical calculations would become prohibitively tedious. This is a limitation of the likelihood method compared to the ECM test.

4. Example

We will illustrate the problem of testing multiple tumors with an example from a prior study of melanoma (Orlow et al., 2009). The data from two patients, one with 3 tumors and another one with 4 tumors, are shown in Table 2.

For patient 1 the pairwise CM tests with continuity correction give p-values equal to 0.007 (Tumors 1 vs 2), 0.013 (1 vs 3) and 0.34 (2 vs 3). It is not obvious in this case whether all 3 tumors or only two should be concluded clonal – there might be not enough power to detect clonality in all 3 pairwise tests. Note that two markers have concordant LOH in all 3 tumors, and the ECM test of all 3 tumors has a p-value of 0.03. The minP adjusted p-values are 0.02 for the set of tumors 1,2,3, 0.012 for 1,2, 0.017 for 1,3, and 0.32 for 2,3, so the global hypothesis of independence is rejected. Since the minP adjusted p-value for the test of all 3 tumors is significant, we conclude that all 3 tumors are clonal. The p-value using the LR test is 0.01. Figure 3a) shows the topology with the maximum likelihood ratio for this patient. Tumors 1 and 2 share a higher clonality signal, and the corresponding pairwise test is most significant.

For patient 2 there are 6 pairwise comparisons: 0.024 (tumors 1 vs 2), 0.024 (1 vs 3), 0.0007 (1 vs 4), 0.003 (2 vs 3), 0.003 (2 vs 4), and 0.003 (3 vs 4). If the Bonferroni correction is applied, the first two comparisons are not significant, and it is not clear how to classify these tumors. The p-values for ECM test for patient 2 are as follows (in increasing order): 3.8×10^{-6} (tumors 2,3,4), 6.7×10^{-6} (1,2,3,4), 4×10^{-4} (1,2,3; 1,3,4), 6.8×10^{-4} (1,4), 2.7×10^{-3} (2,3; 2,4; 3,4), 2.4×10^{-2} (1,2; 1,3). All minP adjusted p-values remain significant except for subsets 1,2 and 1,3 – their p-values become 0.054. Since the intersection of all minP adjusted significant subsets is all 4 tumors, we conclude that the 4 tumors are clonal. The p-value for the LR test is <0.001 and the topology with the maximum likelihood ratio is shown in Figure 3b). All tumors share a pretty strong clonality signal ($\hat{c}$= 0.91), and tumors 1 and 4 are a little bit closer to each other compared to 2 and 3. In the next section we will evaluate how these methods perform in simulations where the true relationship among tumors is known.

5. Simulations

5.1 Testing the global null hypothesis that all tumors are independent using ECM and Likelihood based tests

We tested the accuracy of the proposed methods using the simulation study presented in Table 3. We generated 4 tumors with $J = 15$ markers using two types of LOH frequencies: all $p_i = 0.3$, or 3 groups of 5 markers each having frequencies 0.1, 0.3 or 0.5. Both types of trees from Figure 2 and various clonality signals shown in columns 4–6 of Table 3 were assessed. In all of our simulations we assume $\pi = 0.5$ while both generating the data and estimating likelihoods. For each scenario, 1000 patients were generated.

We have performed ECM testing using all possible tumor subsets, and using only 6 pairwise comparisons, and we assessed two methods of multiplicity adjustment: Bonferroni and minP. The last two columns in Table 3 show performance of the likelihood based method. In first of them we assumed the true frequencies p_i are known, and in the last column we assumed a more realistic setting when the frequencies are known with error, i.e. while LOH had true frequencies p_i, for the likelihood calculation and in generating the reference distribution we used instead $p_i^*: \log(p_i^*/(1-p_i^*)) = \log(p_i/(1-p_i)) + \varepsilon_i$, where $\varepsilon_i \sim N(0, 0.32)$. This variance of error (0.32) was selected because it had been previously observed in real datasets (Ostrovnaya et al., 2008). More details about how the tumor data were generated are given in Appendix A2. Each cell in Table 3 contains the proportion of simulations for which H_0 is rejected at nominal level α=0.05). The rows corresponding to the null hypothesis ($c = c_{12} = c_{34} = c_{234} = 0$) contain type contain type I error. The number in the parentheses represents calibrated power - proportion of simulations for which H_0 is rejected at significance level that will make the actual type I error equal to 0.05.

As shown in Table 3, when $p_i = 0.3$, $i = 1, \ldots, J$, the Bonferroni method has slightly conservative type I error, while the minP correction has type I error close to the nominal level, and both of these methods have similar calibrated power. Regardless of the choice of multiplicity adjustment, combining ECM tests from all possible subsets is more powerful compared to the combination of all pairwise tests, despite more stringent multiplicity adjustment. LR methods and minP adjusted tests of all subsets have comparable power except in scenario 3, where tumors 1 and 2 are clonal yet independent of tumors 3 and 4, which are also clonal. Here minP is less powerful because, unlike the LR method, it includes adjustment for multiple testing. For the LR method, the error in the LOH frequencies only slightly increases the type I error and slightly decreases the power.

When the p_i'*s* are not homogenous, LR still has type I error close to 0.05, and the conservative Bonferroni method achieves similar rate. By contrast minP has significantly higher type I error of 0.12 in those circumstances. The LR methods have slightly higher calibrated power than ECM based methods, presumably since the LR takes into account heterogeneity among markers.

In summary, when LOH frequencies are similar, testing all possible tumor subsets and subsequent minP multiplicity adjustment is the best method among nonparametric strategies considered here, and it has similar power to the LR test. However, if markers have

heterogenous p_i's, then the assumptions of the ECM based methods are violated and the LR method has superior performance. These results are consistent with Ostrovnaya et al., 2008. Note that these scenarios were chosen for comparative purposes, and in real experiments the power will likely be higher for larger J, p_i's or clonality signal.

5.2 Selecting clonal tumors using ECM and LR tests

Table 4 shows how often the correct clonal subset is selected among the cases where the global null hypothesis is rejected. The data generated for Table 3 was used. For the ECM based tests we take the union of all overlapping subsets that have significant adjusted p-values. For the LR test we determine which set of tumors is clonal based on whether the corresponding MLEs of parameters c, c_{12}, c_{34}, c_{234} are greater than 0.1, threshold selected to filter out values close to 0. For example if $\hat{c} \geq 0.1$, then tumors 1,2,3,4 must be clonal, but if $\hat{c} < 0.1$, $\hat{c}_{234} > 0.1$ and the model with the highest likelihood ratio is topology 2, then tumors 2, 3, 4 are clonal. The subset that is truly clonal according to this definition is shown in Table 4.

Methods that use pairwise tests are much less accurate in most scenarios, while there is no clear winner among all other methods. When all 4 tumors are clonal, the clonality signal is strong enough and almost all methods pick it up with high accuracy. When only 2 tumors are clonal, Bonferroni and minP methods outperform LR, and in the remaining scenarios LR method does better. Note that probability to correctly select the clonal subsets is only directly comparable between methods when their power to reject the global null hypothesis is the same, so this table should be interpreted in conjunction with Table 3.

Note that for one patient with 4 tumors and J=15 markers computing minP adjusted p-values using 1000 simulations takes about 2.5 minutes on a computer with 2.33GHz CPU. Computing maximum likelihood ratio takes less than 15 seconds, and it takes 100–1000 times as long to calculate the reference distribution needed for obtaining the p-values.

6. Discussion

We have extended the previously developed methodology for testing clonality of two tumors to testing three and more tumors. These methods are substantially more powerful compared to combined pairwise tumor comparisons. Both parametric and nonparametric frameworks are available. The nonparametric Extended Concordant Mutations test can be performed in any subset of tumors, and the theoretical null distribution for its test statistic, the number of concordant mutations in a set of tumors, is available in closed form. The tests of all possible tumor subsets can be adjusted for multiplicity by minP method utilizing joint distribution of the p-values obtained using permutations. As shown in our simulations, the ECM testing of all subsets of tumors performs substantially better than all possible pairwise tests. The ECM test can be used when markers have similar probabilities of mutation and it is likely that maternal and paternal alleles are equally affected.

The alternative method is based on the likelihood model. It is parameterized to allow different shared clonality signals for different subsets of tumors. LOH frequencies at each marker can also vary but are assumed to be known from the previous studies or estimated

from other patients in the cohort. The imbalance in mutation probability between maternal and paternal alleles can be easily incorporated into the likelihood. The advantage of this method is that only one test for the global null hypothesis is performed. In many scenarios the likelihood based method is more powerful than the ECM testing with multiple comparisons adjustment, and its performance is only slightly worse when the frequencies of mutations are known with error, i.e. when they are estimated based on the small number of samples. If there is a concern that the assumed mutation frequencies have high variance, one can perform the test with several different plausible sets of frequencies and compare the results.

We have explicitly derived the likelihood for 3 and 4 tumors, but extending it to more tumors would be challenging. This is a disadvantage of the LR method compared to the ECM test. When 5 or more tumors are analyzed and mutation probabilities differ substantially across markers, the ECM test, while anticonservative, can still be used with rather stringent significance threshold; alternatively, the LR based test can be applied to all possible quadruples of tumors.

Once the null hypothesis that all the tumors are independent is rejected, it is possible to indentify which subset of tumors is most likely clonal. We offer simple heuristic rules for finding such subset, such as intersecting all overlapping subsets with significant adjusted p-values, or selecting the topology using the maximum likelihood ratio and parameter estimates. These rules allow us to compare different methods, and they seem to work well in simulations. It is possible, however, that in practice few other candidate topologies are nearly as significant as the best one. Thus, researchers might choose to vary the threshold of significance for the ECM test, or take into consideration the likelihood ratios of all topologies. We believe that even though a more formal algorithm that tests whether any topology is more likely than others could potentially be developed, it would have low power given the relatively small number of markers in the typical study.

Note that the likelihood model is specific to the assumed tumor topology, and tumors are not exchangeable. As a result it does not reduce to a single likelihood under the null hypothesis that will be symmetric across tumors. This limitation arises from allowing unequal relationships within different tumor subsets. We can also specify a symmetric model where all 3 or 4 tumors will have exactly the same clonality signal. It would require pooling some outcomes and adding the corresponding likelihood terms. Such a model is likely to have similar properties to the ECM test, i.e. it will be most powerful if the correct subset is tested, and will have similar performance to the ECM test when the markers are exchangeable. We did not pursue this formulation further because it is not straightforward to infer tumor topology from the tests of multiple subsets.

Recently there has been a great deal of interest in comparing copy number arrays of multiple tumor samples from the same patient, or even multiple samples of the same tumor, and building their phylogenetic tree even when it is known that they are clonal (Letouze et al., 2010, Liu et al., 2009). Instead, LOH profiles are easier to obtain and can potentially be used for these purposes. The proposed methodology will facilitate such analyses.

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Appendix

A1. Formulas for the likelihood model of 3 tumors for topology shown in Figure 1.

$$\begin{aligned}
h_i^{\text{“SS,S”}} &= cp_i + \frac{(1-c)p_i c_{12} p_i}{1-cp_i}(\pi^2+(1-\pi)^2) + \frac{(1-c)p_i(1-c-c_{12})^2 p_i^2}{(1-cp_i)(1-(c+c_{12})p_i)}(\pi^3+(1-\pi)^3),\\
h_i^{\text{“SL.S”}} &= \frac{(1-c)p_i(1-c-c_{12})^2 p_i^2}{(1-cp_i)(1-(c+c_{12})p_i)} 2(\pi^2(1-\pi)+\pi(1-\pi)^2),\\
h_i^{\text{“SS,L”}} &= \frac{(1-c)p_i c_{12} p_i}{1-cp_i}(2\pi(1-\pi)) + \frac{(1-c)p_i(1-c-c_{12})^2 p_i^2}{(1-cp_i)(1-(c+c_{12})p_i)}(\pi^2(1-\pi)+\pi(1-\pi)^2),\\
h_i^{\text{“S0,L”}} &= \frac{(1-c)p_i(1-p_i)(1-c-c_{12})p_i}{(1-cp_i)(1-(c+c_{12})p_i)}(4\pi(1-\pi)),\\
h_i^{\text{“SL,0”}} &= \frac{(1-p_i)(1-c-c_{12})^2 p_i^2}{(1-cp_i)(1-(c+c_{12})p_i)}(2\pi(1-\pi)),\\
h_i^{\text{“S0,S”}} &= \frac{(1-c)p_i(1-p_i)(1-c-c_{12})p_i}{(1-cp_i)(1-(c+c_{12})p_i)}(2(\pi^2+(1-\pi)^2)),\\
h_i^{\text{“SS,0”}} &= \frac{(1-p_i)c_{12}p_i}{1-cp_i} + \frac{(1-p_i)(1-c-c_{12})^2 p_i^2}{(1-cp_i)(1-(c+c_{12})p_i)}(\pi^2+(1-\pi)^2),\\
h_i^{\text{“S0,0”}} &= \frac{(1-p_i)^2(1-c-c_{12})p_i}{(1-cp_i)(1-(c+c_{12})p_i)} 2,\\
h_i^{\text{“00,S”}} &= \frac{(1-p_i)^2(1-c)p_i}{(1-cp_i)(1-(c+c_{12})p_i)},\\
h_i^{\text{“00,0”}} &= \frac{(1-p_i)^3}{(1-cp_i)(1-(c+c_{12})p_i)}.
\end{aligned}$$

A2. The details of data generation for simulations in Tables 3 and 4.

For tumors with topology 1 the common clonal LOH events were generated with probability cp_i, the subsequent clonal mutations shared by tumors 1 and 2 (3 and 4), given that no common event has occurred, were generated with probability $\frac{c_{12}p_i}{1-cp_i}$ ($\frac{c_{34}p_i}{1-cp_i}$), while all subsequent independent events were generated with probability $\frac{(1-c-c_{12})p_i}{1-(c+c_{12})p_i}$ ($\frac{(1-c-c_{34})p_i}{1-(c+c_{34})p_i}$). For tumors with topology 2 the common clonal LOH events were generated with probability cp_i, the subsequent clonal mutations shared by tumors 2,3 and 4, given that no common event has occurred, were generated with probability $\frac{c_{234}p_i}{1-cp_i}$, the subsequent clonal mutations shared by tumors 3 and 4 only were generated with probability $\frac{c_{34}p_i}{1-(c+c_{234})p_i}$, while independent events in tumors 1, 2 or 3,4 were generated with probabilities $\frac{(1-c)p_i}{1-cp_i}$, $\frac{(1-c-c_{234})p_i}{1-(c+c_{234})p_i}$, or $\frac{(1-c-c_{234}-c_{34})p_i}{1-(c+c_{234}+c_{34})p_i}$.

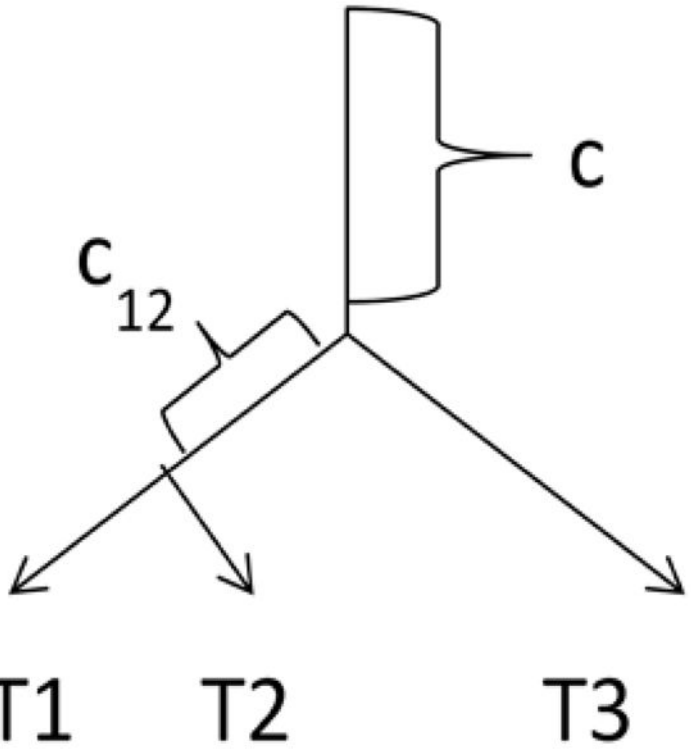

Figure 1.
Tree topology for 3 tumors.

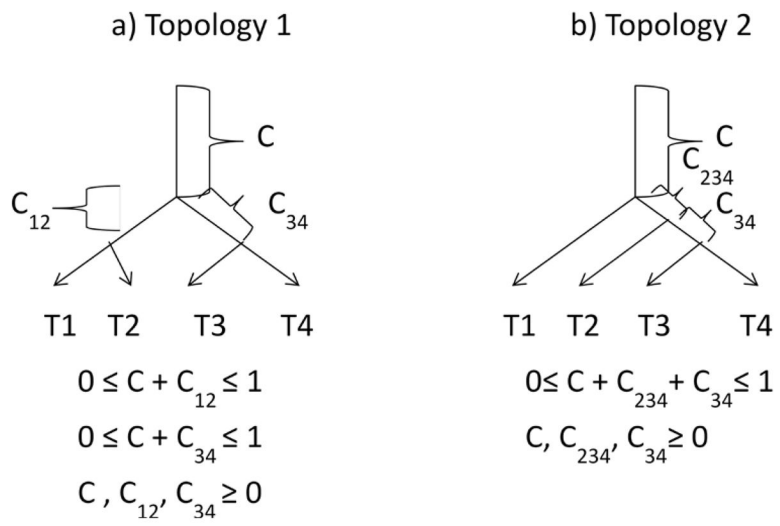

Figure 2.
Tree topologies for 4 tumors.

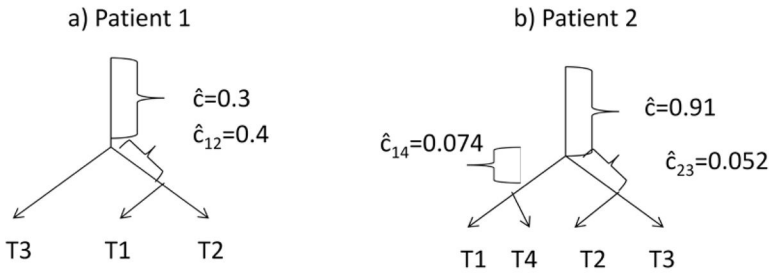

Figure 3.
Estimated trees for data in the examples from Section 4.

Table 1

Simulation study of power and type I error of ECM test.

p_i	c	clonal tumors	ECM_{1234}	ECM_{123}	ECM_{124}	ECM_{134}	ECM_{12}	ECM_{13}	ECM_{14}	ECM_{2}
0.3	0	none	0.01	0.05	0.03	0.04	0.05	0.03	0.03	0.04
0.3	0.5	1,2	0.07	0.18	0.15	0.04	0.63	0.04	0.03	0.03
0.3	0.5	1,2,3	0.29	0.90	0.16	0.16	0.63	0.62	0.04	0.64
0.3	0.5	1,2,3,4	0.89	0.88	0.88	0.88	0.61	0.60	0.59	0.59

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Table 2

Example of LOH profiles of multiple tumors.

		Patient 1			Patient 2			
	%LOH	T 1	T 2	T 3	T 1	T 2	T 3	T 4
D1S214	0.58	0	0	0	-	-	-	-
D1S2766	0.39	-	-	-	0	0	0	0
D1S2882	0.62	L	L	L	0	L	0	0
D2S131	0.98	-	-	-	0	0	0	0
D2S139	0.50	L	L	0	0	0	S	0
D2S2182	0.58	L	0	L	0	0	0	0
D2S206	0.47	0	0	0	x	x	x	x
D3S1293	0.39	0	0	0	0	0	0	0
D4S1543	0.62	0	0	0	-	-	-	-
D6S1043	0.69	0	0	0	-	-	-	-
D6S275	0.76	S	0	0	S	S	S	S
D6S457	0.69	0	0	S	x	x	x	x
D7S1824	0.71	0	L	S	0	0	0	0
D8S1104	0.27	0	0	L	-	-	-	-
D9S157	0.55	L	L	L	S	0	0	S
D10S212	0.54	0	S	0	S	S	S	S
D10S676	0.70	L	L	0	-	-	-	-
D11S199	0.58	-	-	-	0	0	0	0
D11S200	0.82	S	0	S	0	S	S	S
D13S153	0.95	-	-	-	0	0	0	0
D17S786	0.58	0	0	L	0	0	0	0
TP53	0.65	L	0	L	-	-	-	-
D17S132	0.58	0	0	S	-	-	-	-

Notations: “-” – uninformative marker; “0” – informative marker, no LOH; S and L – LOH at opposite alleles; x – marker is excluded from consideration to maintain the assumption of independence between markers; % LOH – frequency of LOH at these markers in the remaining 22 melanoma patients in Orlow et al., (2009).

Table 3

Power and test size for testing global null hypothesis that all tumors are independent.

							ECM based methods				Likelihood based methods	
Scenario	**Topology**	p_i	c	c_{12}	c_{34}	**True clonal set**	**Bonferroni**	**Bonferroni - pairwise**	**minP**	**minP pairwise**	**LR**	**LR (wrong p)**
1	1	0.3	0	0	0	None	0.02 (0.05)	0.02 (0.05)	0.04 (0.05)	0.04 (0.05)	0.04 (0.05)	0.06 (0.05)
2	1	0.3	0	0.5	0	12	0.27 (0.46)	0.34 (0.44)	0.41 (0.44)	0.41 (0.46)	0.44 (0.47)	0.47 (0.43)
3	1	0.3	0	0.5	0.5	12,34	0.47 (0.69)	0.56 (0.7)	0.65 (0.68)	0.68 (0.72)	0.76 (0.78)	0.78 (0.76)
4	1	0.3	0.5	0	0	1234	0.88 (0.91)	0.61 (0.71)	0.91 (0.91)	0.7 (0.74)	0.92 (0.93)	0.92 (0.92)
5	1	0.3	0.5	0	0.3	1234	0.92 (0.94)	0.83 (0.9)	0.94 (0.94)	0.88 (0.9)	0.96 (0.96)	0.96 (0.96)
6	1	0.1,0.3,0.5	0	0	0	None	0.06 (0.05)	0.06 (0.05)	0.12 (0.05)	0.12 (0.06)	0.06 (0.05)	0.07 (0.05)
7	1	0.1,0.3,0.5	0	0.5	0	12	0.34 (0.3)	0.41 (0.38)	0.51 (0.33)	0.53 (0.38)	0.45 (0.41)	0.43 (0.38)
8	1	0.1,0.3,0.5	0	0.5	0.5	12,34	0.56 (0.5)	0.61 (0.58)	0.71 (0.53)	0.71 (0.59)	0.73 (0.69)	0.71 (0.67)
9	1	0.1,0.3,0.5	0.5	0	0	1234	0.89 (0.87)	0.68 (0.65)	0.91 (0.88)	0.76 (0.65)	0.89 (0.88)	0.89 (0.87)
10	1	0.1,0.3,0.5	0.5	0	0.3	1234	0.95 (0.93)	0.86 (0.83)	0.97 (0.93)	0.91 (0.84)	0.97 (0.96)	0.96 (0.96)
			c	c_{234}	c_{34}							
11	2	0.3	0	0.2	0.3	234	0.39 (0.56)	0.36 (0.48)	0.52 (0.56)	0.45 (0.49)	0.55 (0.58)	0.6 (0.57)
12	2	0.3	0.3	0.2	0.3	1234	0.9 (0.94)	0.84 (0.89)	0.94 (0.94)	0.89 (0.9)	0.96 (0.96)	0.96 (0.95)
13	2	0.1,0.3,0.5	0	0.2	0.3	234	0.46 (0.42)	0.41 (0.38)	0.58 (0.43)	0.51 (0.38)	0.53 (0.48)	0.51 (0.45)
14	2	0.1,0.3,0.5	0.3	0.2	0.3	1234	0.9 (0.88)	0.84 (0.83)	0.94 (0.89)	0.89 (0.83)	0.95 (0.94)	0.94 (0.92)

Table 4

Percent of patients with correctly identified clonal tumor subset among those patients for whom the global null hypothesis was rejected.

							ECM based methods				Likelihood based methods	
Scenario	**Topology**	p_i	c	c_{12}	c_{34}	**True clonal set**	**Bonferroni**	**Bonferroni - pairwise**	**minP**	**minP pairwise**	**LR**	**LR (wrong p)**
2	1	0.3	0	0.5	0	12	0.81	0.95	0.70	0.89	0.41	0.35
3	1	0.3	0	0.5	0.5	12,34	0.08	0.19	0.14	0.25	0.70	0.67
4	1	0.3	0.5	0	0	1234	1.00	0.51	0.99	0.64	1.00	1.00
5	1	0.3	0.5	0	0.3	1234	0.94	0.40	0.95	0.54	0.97	0.96
7	1	0.1,0.3,0.5	0	0.5	0	12	0.61	0.85	0.55	0.77	0.39	0.37
8	1	0.1,0.3,0.5	0	0.5	0.5	12,34	0.10	0.21	0.14	0.27	0.65	0.63
9	1	0.1,0.3,0.5	0.5	0	0	1234	0.98	0.52	0.98	0.65	0.98	0.98
10	1	0.1,0.3,0.5	0.5	0	0.3	1234	0.93	0.42	0.95	0.55	0.96	0.96
			c	c_{234}	c_{34}							
11	2	0.3	0	0.2	0.3	234	0.52	0.14	0.46	0.20	0.53	0.50
12	2	0.3	0.3	0.2	0.3	1234	0.69	0.12	0.77	0.25	0.75	0.76
13	2	0.1,0.3,0.5	0	0.2	0.3	234	0.45	0.11	0.39	0.20	0.49	0.48
14	2	0.1,0.3,0.5	0.3	0.2	0.3	1234	0.72	0.19	0.79	0.32	0.72	0.75