A classification model for distinguishing copy number variants from cancer-related alterations

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Abstract

Both somatic copy number alterations (CNAs) and germline copy number variants (CNVs) that are prevalent in healthy individuals can appear as recurrent changes in comparative genomic hybridization (CGH) analyses of tumors. In order to identify important cancer genes CNAs and CNVs must be distinguished. Although the Database of Genomic Variants (Iafrate et al., 2004) contains a list of all known CNVs, there is no standard methodology to use the database effectively.

We develop a prediction model that distinguishes CNVs from CNAs based on the information contained in the Database and several other variables, including potential CNV's length, height, closeness to a telomere or centromere and occurrence in other patients. The models are fitted on data from glioblastoma and their corresponding normal samples that were collected as part of The Cancer Genome Atlas project and hybridized on Agilent 244K arrays. Using the Database alone CNVs can be correctly identified with about 85% accuracy if the outliers are removed before segmentation and with 72% accuracy if the outliers are included, and additional variables improve the prediction by about 2-3% and 12%, respectively.
A classification model for distinguishing copy number variants from cancer-related alterations

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Abstract

Motivation

Both somatic copy number alterations (CNAs) and germline copy number variants (CNVs) that are prevalent in healthy individuals can appear as recurrent changes in comparative genomic hybridization (CGH) analyses of tumors. In order to identify important cancer genes CNAs and CNVs must be distinguished. Although the Database of Genomic Variants (Iafrate et al., 2004) contains a list of all known CNVs, there is no standard methodology to use the database effectively.

Results

We develop a prediction model that distinguishes CNVs from CNAs based on the information contained in the Database and several other variables, including potential CNV’s length, height, closeness to a telomere or centromere and occurrence in other patients. The models are fitted on data from glioblastoma and their corresponding normal samples that were collected as part of The Cancer Genome Atlas project and hybridized on Agilent 244K arrays. Using the Database alone CNVs can be correctly identified with about 85% accuracy if the outliers are removed before segmentation and with 72% accuracy if the outliers are included, and additional variables improve the prediction by about 2-3% and 12%, respectively.

1 Introduction

Copy number variants (CNVs) are a recently discovered part of natural genetic variation in humans. CNVs, also sometimes known as copy number variations or copy number polymorphisms, is a collective term for deletions, insertions, duplications and large-scale copy number variants ranging in size between one kilobase and several megabases (Iafrate et al., 2004, Freeman et al., 2006, Redon et al., 2006, Carter, 2007). About 15% of the human genome, including thousands of genes, may be variable in copy number, and this variation can be *de novo* (occurring for the first time in the parent’s germ cell) or inherited from the parents by healthy individuals (de Smith et al., 2008). Although their significance is not fully understood, it is likely that CNVs are responsible for a considerable part of phenotypic variation. For example, there are established links between CNVs and childhood onset of schizophrenia (Walsh et al., 2008) and autism (Sebat et al., 2007). It has also been shown that CNVs can increase the risk of prostate cancer (Liu et al., 2009) and neuroblastoma (Diskin et al., 2009). CNVs can contribute to our understanding of complex diseases through genome-wide association studies together with SNPs and other types of variants (Beckmann et al., 2007, Ionita-Laza et al., 2009). DNA copy number arrays are the main instruments for identifying CNVs. The constant refinement and increasing resolution of these assays are helping to discover and map many of these variants with high precision.

Before the importance of CNVs was realized it was well known that copy number changes occur often in cancer. Throughout the manuscript we call such changes copy number alterations (CNAs). CNAs are somatic changes in genomic copy number of any size, up to a whole chromosome, that occur in the genome of a cancerous cell. These changes often involve important cancer genes such as tumor suppressor genes and oncogenes. To find important cancer genes, investigators look for regions that are repeatedly gained or lost in patients with a particular cancer. CNVs, present in the genome of every cell, are also present in tumor cells. Thus, when comparative genomic hybridization (CGH) arrays of tumors are studied both cancer-related CNAs and germline CNVs can appear as unique or recurrent changes. It would be possible to avoid CNVs using a paired tumor-normal design, with the normal tissue from the same individual used as a reference. However, if a paired normal sample is not used or is not available, which is often the case, it is difficult to distinguish CNAs from CNVs.

To our knowledge, there are currently no statistical methods to identify CNVs in tumor data. A common practice, recommended, for example, in (Cho et al., 2006), is to evaluate whether the discovered recurrent regions match known CNVs in the Database of Genomic Variants (http://projects.tcag.ca/variation) (Iafrate et al., 2004), which we call the
DGV. It is unclear, however, to what extent the regions should match known CNVs. The problems associated with this practice are that it might exclude unnecessarily big regions of the genome, and that regions of exclusion are imprecise due to uncertainty in the endpoints of the known CNVs (Cho et al., 2006). In addition, the smaller CNVs are less likely to be included into the DGV since they are detected less frequently in older, low-resolution arrays.

Our goals in this manuscript are twofold. Our first goal is to determine if additional information can be added to that from the DGV for predicting CNVs in cancer data. Our second goal is to determine how to optimally use the DGV for this same purpose. We are looking for any characteristics of CNV regions that are different from cancer-modified segments and thus can be used to improve prediction. We develop statistical models toward this goal. These models, in addition to simplifying data analysis, might allow the discovery of new CNVs from the abundance of available tumor data.

In order to develop our models we have used data from the Agilent 244K array (AG244) collected on glioblastoma (GBM) patients as part of The Cancer Genome Atlas (TCGA) project (TCGA-Network, 2008). TCGA data are informative because they include many paired tumor and normal tissue samples that have been hybridized in the same manner. Specifically, the tumor samples have been hybridized against a single reference sample, and the matching normal samples have been hybridized against the same reference sample. Thus one can identify CNVs in the normal samples as well as distinguish between CNAs and CNVs in the tumor samples. We investigate whether CNVs and CNAs differ in such variables as length, height, loss/gain status, overlap with changes in other patients, and other variables. We divide the patients into training and validation sets, and develop a prediction model that includes these variables and known literature on CNVs. In addition, TCGA has collected copy number data on several normal tissue samples, which should contain only CNVs, and data from tumor samples co-hybridized with the patient’s own normal tissue reference, which should contain only CNAs. We use these data to further validate our models.

The outline of the paper is as follows. In the next section we describe the data and introduce the possible variables in the model. Section 3 contains the quantitative results for the main effects of the predictors, fitted models and their accuracy. The summary and discussion follow in Section 4.

2 Methods

2.1 Selection of patients

We initially selected the first 206 glioblastoma samples qualified for genomic analyses as part of TCGA (TCGA-Network, 2008). All of them were hybridized to the AG244 array as well as other platforms. Here, we limit our analyses to AG244 data collected at the Memorial Sloan-Kettering Cancer Center. There were 78 patients that satisfied requirements for our analysis: paired tumor and normal samples that were independently hybridized against pooled reference. The normal samples varied between blood, skin, and muscle tissue. The patient data were divided into training and test sets by TCGA batch number in order to
maintain the heterogeneity that is likely to occur in practice. The training set consisted of TCGA batches 3, 6, and 7, with 43 patients, while the test set contained batch 5 with 35 patients. The data are of exceptionally high quality and publicly available at (http://cancergenome.nih.gov). The details of the sample selection and preparation are given in (TCGA-Network, 2008).

We originally chose glioblastoma samples to study CNVs because it was the only set of TCGA patients with a large enough set of matched samples. As it turns out, glioblastoma is a disease where distinguishing germline mutations might prove to be particularly useful because tumor induced alterations are often of similar size. Among heterozygous deletions and amplifications that are present in GBM in at least 10% of patients, as reported previously (Kotliarov et al., 2006), 40% are focal alterations (1 basepair), and about 90% of the rest of them are under 3 Mb. Altered regions of similar sizes are reported in the TCGA manuscript (TCGA-Network, 2008). In fact its authors excluded regions as CNVs if they 1) appeared to be CNVs in HapMap normals, or 2) appeared in at least 2 independent publications in DGV, or 3) appeared in the matched normal tissue by manual or automated search. These exclusion criteria are quite complicated and hard to replicate. We develop a model that would simplify such an analysis.

2.2 Segmentation analysis

The raw log-ratio data were normalized as described in the supplementary section of the TCGA manuscript (TCGA-Network, 2008). To identify possible regions of gain and loss, we segmented the normalized log ratios using two different algorithms: CBS and GLAD. CBS, or Circular Binary Segmentation (Olshen et al., 2004, Venkatraman and Olshen, 2007) is a method for segmenting data into regions of equal estimated copy number. It has been found to have good properties compared to other similar methods (see Willenbrock and Fridlyand, 2005, Lai et al., 2008), and is included in the Bioconductor package DNAcopy (www.bioconductor.org). We have used all default parameters, including the significance level of $\alpha = 0.01$, except for the minimum gap between the segments was set to be one standard deviation (undo.sd=1). Users frequently apply a smoothing procedure to remove outliers from the data prior to segmentation, which can increase power and remove some smaller gains and losses. On the other hand, smaller regions eliminated by smoothing may contain CNVs. Therefore, we fit the prediction model on both smoothed and non-smoothed data.

Alternatively, we estimated intervals of change using the GLAD algorithm (Hupe et al., 2004, Bioconductor package GLAD) with default parameters. GLAD automatically filters outliers, analogous to smoothing in CBS. The second method was used to ensure that the accuracy of the prediction model was not specific to a particular segmentation method.

2.3 Candidate CNVs

The unit of analysis for us is a segment of constant copy number with breakpoints estimated by one of the segmentation algorithms previously described. We are calling any segment a
gain or loss that has an average log ratio of at least one median absolute deviation above or below the array’s median, respectively. Segments inside this range are called normal.

Suppose only tumor data were available. Then long enough gains and losses would not be confused with CNVs, since CNVs are not longer than several Megabases. Likewise, if a segment of interest is in the midst of larger gains or losses, it is less important to identify whether this particular segment was modified in the germline. Therefore, we consider every segment of gain or loss in the tumor that has length of up to 2.3 Megabases AND is flanked by at least one normal segment to be a candidate CNV for our classification model. This definition reproduces the situation where the question of identifying CNVs might arise.

Consecutive gains or consecutive losses were combined if their total length was under 2.3 Mb. The upper threshold for length was motivated by the analysis of true CNVs from the normal samples. Note that CNVs of greater length than this are reported in the literature; however, they comprise less than 1% of reported CNVs, and might have characteristics vastly different from the majority. Although by formal definition CNVs have to be at least 1kb in length as stated by (Redon et al., 2006), we did not use this restriction. Since the gap between probes was often large and segment lengths were possibly underestimated, we have included the segments that were shorter than 1Kb, but there were only few of them.

Chromosomes X and Y were excluded from consideration. Any candidates located in the "physiological" regions shown in Table 6 were also excluded following suggestion by (Scherer et al., 2007). Physiologic CNVs reflect normal somatic rearrangements that occur in the immunoglobulin genes and T-cell receptors during their development (Chowdhury and R, 2004, Belessiand et al., 2006, Bemark and MS, 2003).

The matching normal samples were processed and segmented in exactly the same fashion as the tumor samples. They were used to determine which of the candidate CNVs were true CNVs. For example, consider Figure 1. All the red segments of gain and loss were found by smoothed CBS on one chromosome, in addition the blue segments were found if the data were non-smoothed. The top and bottom panels represent a tumor and the corresponding normal tissue, respectively. If a gain or loss in a tumor sample exactly matched a gain or loss in a normal sample, it was considered a true CNV. However, the segmentation algorithm introduced error in estimation of the breakpoints, so even true CNVs might not exactly match between the samples. For example, the red segment marked as CNV overlapped with the matching loss in the normal sample that did not have exactly the same breakpoints.

Furthermore, some segments in the normal sample might not make it to the required threshold of significance and thus would not be identified by the segmentation algorithm. In the figure it can be seen that there is an extremely low log ratio (outlier) in a normal sample corresponding to the last candidate loss (in blue) in the tumor, but it is not extreme or long enough to be identified by CBS. To make sure that such segments were not missed we perform a conditional segmentation test for each candidate CNV. In this test we consider log-ratios in the normal sample corresponding to the candidate segment in a tumor. Suppose there are k of them. First we calculate their mean $\mu$. Then we randomly draw k values from the pool of normal sample log-ratios that are located within the candidate and two of its neighboring segments in the tumor and calculate their mean $\mu^\ast$; this procedure is repeated
Figure 1: Example of CNVs and CNAs. One chromosome is shown. Segments in blue are found only in unsmoothed data. The upper panel contains tumor, while the lower panel is a matching normal sample. CNVs have either a matching segment in the normal sample identified by a segmentation algorithm or matching significantly extreme log-ratios identified by a permutation test. Regions of the normal sample corresponding to CNAs are normal.
1000 times. If the mean of normal log-ratios corresponding to the candidate segment $\mu$ exceeds the gain/loss threshold and is less extreme than the simulated means $\mu^*$ in less then ($\alpha=0.01 \times 100$)% of draws, we declare the candidate to be a true CNV.

In summary, each gain or loss candidate CNV is considered a true CNV if it overlaps a gain or loss in a normal sample identified by a segmentation algorithm, or if the mean of the corresponding segment in the normal sample is significant by the conditional segmentation algorithm described above. Otherwise the candidates are considered to be CNAs.

The blue segments in Figure 1 illustrate the trade-offs associated with unsmoothed data. On one hand, one additional CNA and three CNVs emerge when outliers are included in the segmentation. On the other hand, some of these new candidates appear as a result of just one extreme marker. Unless there are array artifacts, it is unlikely that the outliers will simultaneously occur in two arrays in exactly the same probe, and that gives us confidence that unsmoothed data can be useful.

True CNV status for each candidate CNV is a response variable in our model. The predictors for our model are discussed below.

2.4 Database of genomic variants

As mentioned before, previously discovered CNVs are reported in the DGV. This database has been used before to distinguish CNAs from CNVs (e.g. TCGA-Network [2008]), but there is no standard quantitative way to make this distinction. In addition, there is some uncertainty in the breakpoints in the reported CNVs. We propose to quantify the overlap with the DGV in the following way. For each probe we calculate the total number of reported variants (unique Variation IDs) that include it. Then, for each candidate CNV its Database score is defined as average number of these reported variants across all the probes that comprise it. The resulting number is not usually an integer since most overlapping regions in the database do not have the same breakpoints. The less a candidate segment overlaps known CNVs in the DGV, the smaller its Database score.

Alternatively, instead of the number of variants we used the total reported number of people that had variants overlapping a probe, and this score is called Database score II. This variable is potentially informative because each variant from the DGV has different frequency.

The DGV is updated regularly. For our analysis, we used version 7 of the database for genome build 'hg18' from March 2009 available at http://projects.tcag.ca/variation/downloads/variation.hg18.v7.mar.2009.txt.

2.5 Predictors

Detailed definitions of the candidate predictors for our models are presented in Table 1. These predictors were derived from published results, biological intuition and observations from studying the data. We divide predictors in three categories: "demographic", "derived" and "spatial".
"Demographic" variables are the basic characteristics of the candidate segment, such as its length (in bases), absolute value of segment mean (raw or adjusted by the noise level), gain or loss status, and difference between segment means of two nearby segments adjusted by the noise level. We have also included indicator variables for whether a candidate is surrounded by all normal segments, is within 2 MB of centromere or telomere (Nguyen et al., 2006), or overlaps any of the known areas of segmental duplication (Sharp et al., 2005).

A "derived" variable uses information from arrays of other independent patients of the same study. The most important of these variables records what percent of other tumors also have a candidate CNV in the same location, and whether it is a gain or a loss. Since the breakpoints are estimated with error we used two versions of the same predictor, counting other patients with the segments that either overlap the candidate segment or have at least one of the breakpoints exactly matching. If other patients have both gains and losses in the same location, it is likely that this alteration is a CNV. Similarly, a segment that is in an area where large non-candidate gains (or losses) are frequent is likely to be a CNA.

A "spatial" variable captures possible association of CNVs with other segments located on the same chromosome such as percentage of the chromosome that is gained or lost, existence of nearby candidates and average Database score of the other candidates on the same chromosome.

### 2.6 Statistical methods

The response variable in our model is binary, whether a candidate region is a CNV or CNA, while predictors are either binary, multi-level factors, or continuous. To examine the univariate relationship between CNV status and the predictors we utilized univariate logistic regression. This was because it can accommodate variables of all types and provides us with both significance levels and estimates of the effects. Multivariate logistic regression, however, was not used since many of the predictors were highly collinear.

Classification and Regression Trees (CART) is a suitable alternative for highly collinear data (Breiman et al., 1984). CART is a binary tree approach that is based on recursively splitting on the most predictive variable. CART trees are simple to interpret and have the ability to uncover complex relationships among correlated predictors. One of the disadvantages of CART is that it is often not optimal in terms of prediction error, partly because it is greedy (no looking ahead before splitting). Therefore, we have also used random forests (RF) (Breiman, 2001). RF are a modification of CART that overcome CART’s search difficulties by building multiple trees based on resampling cases. Classification is based on the “votes” of each of these trees. These trees further differ from CART trees because only a random set of predictors is considered at each split. Although this algorithm tends to lead to prediction accuracy that is superior to CART (Breiman, 2001), the results of RF are more difficult to visualize and interpret. Therefore, we used both CART and RF. CART and RF were implemented using the R packages rpart and randomForest, respectively. CART models were pruned according to the ”1-SE" rule.
<table>
<thead>
<tr>
<th><strong>Variable</strong></th>
<th><strong>Definition</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>length of a segment in bases</td>
</tr>
<tr>
<td>Segmental duplication</td>
<td>1 if the candidate is overlapping known region of segmental duplication, 0 otherwise. All regions listed in (Sharp et al., 2006) that could be successfully translated into hg18 by hgLiftOver utility (<a href="http://genome.ucsc.edu/cgi-bin/hgLiftOver">http://genome.ucsc.edu/cgi-bin/hgLiftOver</a>) were used, see Table 7</td>
</tr>
<tr>
<td>Closeness to centromere</td>
<td>1 if the candidate endpoints are within 2Mb of the centromere, 0 otherwise</td>
</tr>
<tr>
<td>Closeness to telomere</td>
<td>1 if the candidate endpoints are within 2Mb of the telomere, 0 otherwise</td>
</tr>
<tr>
<td>Sign</td>
<td>1 if the candidate is a gain, -1 if it is a loss</td>
</tr>
<tr>
<td>Height</td>
<td>absolute value of the candidate segment mean</td>
</tr>
<tr>
<td>Relative height</td>
<td>absolute value of the candidate segment mean divided by the median absolute deviation of the array residuals</td>
</tr>
<tr>
<td>Break</td>
<td>absolute difference between means of two segments surrounding the candidate divided by the median absolute deviation of the array residuals</td>
</tr>
<tr>
<td>Surrounded by Normals</td>
<td>1 if both surrounding intervals are normals, 0 if one of them is a gain or a loss</td>
</tr>
<tr>
<td>Overlap with other patients</td>
<td>factor with levels: GG if there is one or more other patients in the cohort that have overlapping candidates, all of them are gains; LL if there is one or more other patients in the cohort that have overlapping candidates, all of them are losses; GL if there are at least two patients with overlapping candidates, some of them are gains and some are losses; None if there is no other patients with overlapping candidates</td>
</tr>
<tr>
<td><strong>Overlap with other patients</strong> - percent</td>
<td>proportion of other patients in the cohort that have overlapping candidate</td>
</tr>
<tr>
<td>Matching breakpoint in other patients - percent</td>
<td>proportion of other patients in the cohort that have a candidate with at least one exactly matching breakpoint</td>
</tr>
<tr>
<td>Close to other candidates</td>
<td>1 if there is another candidate CNV within 500kb on the same chromosome in this patient</td>
</tr>
<tr>
<td>Percent of Normal</td>
<td>percent of markers on a chromosome where candidate is located that are not lost or gained</td>
</tr>
<tr>
<td>Database score of other candidates</td>
<td>average Database score of other candidates on the same chromosome</td>
</tr>
<tr>
<td>Overlap with CNAs</td>
<td>number of other patients that have overlapping non-candidate segment of the same sign as the candidate (gain or loss)</td>
</tr>
</tbody>
</table>
3 Results

All the analyses were performed on three data sets: smoothed CBS, GLAD and unsmoothed CBS. In the training data set they contained 1448, 1624 and 2037 candidate segments, respectively, and 904 (62%), 744 (46%) and 1448 (71%) of them were considered true CNVs. The samples in the test set accounted for 1683, 1738 and 2686 candidates and 939 (56%), 846 (49%) and 1727 (64%) true CNVs respectively. There are 638 (510) and 761 (674) segments in the training and test set respectively that are true CNVs (true CNAs) in both smoothed CBS and GLAD. The training set contained more patients than the test set but it contained fewer candidate segments. This can be explained by the fact that training set had slightly noisier arrays (higher MAD of residuals), and, therefore, there was less power to detect smaller segments.

3.1 Univariate results

To test association of predictors with true CNV status we pooled the training and test sets. The results within these sets separately were very similar and are not presented. Table 2 contains both Anova p-values and regression $\beta$ coefficients.

The smoothed CBS and GLAD had very similar rankings of significant predictors and their effects. As expected, Database score had the most significant p-value, followed by Matching breakpoint in other patients - percent in CBS or Overlap with other patients - percent in GLAD, Length, and Percent of Normal. All other predictors were also significant. Obviously, overlap with many variants from the DGV was a strong positive predictor of being a CNV. Segments that were shorter, matched with candidates from many other patients or overlapped with both gain and loss candidates in other patients were also more likely to be CNVs. Having other patients with overlapping candidate losses only was also a positive predictor. As seen from the direction of the main effects in Table 2, CNVs also tended to have larger absolute values of segment means; were often surrounded by Normal segments; located on chromosomes with fewer gains and losses, or with other candidates with high Database score; or located close to a telomere, centromere or segmental duplication. Also, we saw several clusters of small CNAs right next to each other, so presence of other candidate segments within 500kb was predictive of CNA.

In unsmoothed CBS the strongest predictor was Overlap with other patients, followed by the Database score. One possible explanation for this difference is that the small CNVs are underrepresented in the DGV but are likely to appear in the unsmoothed arrays of other patients in the cohort. The other notable difference with smoothed segmentation is that closeness to a centromere, telomere or segmental duplication were not significant, possibly because longer CNVs tend to be located there. In fact, the interaction term between length and closeness to centromere (or segmental duplication) was significant in logistic regression for both smoothed and unsmoothed CBS. As demonstrated by the interaction effect, segments at these locations and of longer length were even more likely to be CNVs.

Note that these associations are not causal, and the mechanisms by which CNVs occur and fixate in the population are still to be elucidated.

http://biostats.bepress.com/mskccbiostat/paper17
Table 2: Univariate results by logistic regression, training and test sets combined

<table>
<thead>
<tr>
<th></th>
<th>Smoothed CBS</th>
<th>GLAD</th>
<th>Unsmoothed CBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \beta )</td>
<td>( P )</td>
<td>( \beta )</td>
</tr>
<tr>
<td>Height</td>
<td>3.95E-01</td>
<td>1.16E-20</td>
<td>4.88E-01</td>
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<td>Relative height</td>
<td>7.14E-02</td>
<td>3.36E-17</td>
<td>9.34E-02</td>
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<td>Break</td>
<td>-3.43E-01</td>
<td>1.06E-24</td>
<td>-3.39E-01</td>
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<tr>
<td>Close to other candidates</td>
<td>-1.19E+00</td>
<td>2.17E-32</td>
<td>-8.06E-01</td>
</tr>
<tr>
<td>Overlap with CNAs</td>
<td>-7.10E-02</td>
<td>1.24E-26</td>
<td>-6.67E-02</td>
</tr>
<tr>
<td>Database score</td>
<td>3.06E-01</td>
<td>4.08E-306</td>
<td>3.06E-01</td>
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<tr>
<td>Database score II</td>
<td>9.79E-03</td>
<td>3.19E-159</td>
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<tr>
<td>Overlap w. other pts: %</td>
<td>8.89E+00</td>
<td>4.98E-254</td>
<td>8.02E+00</td>
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<tr>
<td>Matching bkpt in other: %</td>
<td>17.31</td>
<td>2.58E-276</td>
<td>13.13</td>
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<tr>
<td>Overlap w. other pts - GG</td>
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<td>3.63E-199</td>
<td>3.37E-01</td>
</tr>
<tr>
<td>LG</td>
<td>2.85E+00</td>
<td>1.73E+00</td>
<td>2.85E+00</td>
</tr>
<tr>
<td>LL</td>
<td>1.68E+00</td>
<td>2.55E-09</td>
<td>7.98E-01</td>
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<tr>
<td>Closeness to centromere</td>
<td>8.41E-01</td>
<td>2.15E-04</td>
<td>5.78E-01</td>
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<tr>
<td>Closeness to telomere</td>
<td>-2.46E-06</td>
<td>2.14E-130</td>
<td>-1.78E-06</td>
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<tr>
<td>Length</td>
<td>5.98E-02</td>
<td>6.69E-17</td>
<td>5.45E-02</td>
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<td>Dat. score of other cand.</td>
<td>3.40E+00</td>
<td>5.29E-59</td>
<td>2.70E+00</td>
</tr>
<tr>
<td>Percent of Normal</td>
<td>6.81E-01</td>
<td>5.71E-11</td>
<td>7.16E-01</td>
</tr>
<tr>
<td>Segmental duplication</td>
<td>-2.74E-01</td>
<td>5.10E-14</td>
<td>-2.62E-01</td>
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<tr>
<td>Surrounded by Normals</td>
<td>1.30E+00</td>
<td>2.28E-23</td>
<td>1.07E+00</td>
</tr>
</tbody>
</table>
3.2 Prediction models

We have fitted prediction models using smoothed CBS, GLAD and unsmoothed CBS with 4 different sets of predictors. Accuracy was evaluated on 3 validation sets. The full set of predictors contained all the variables described in Table 2 except Database score II and Height that were nearly equivalent to the already included variables Database score and Relative height.

We first discuss the results based on smoothed CBS. The fitted CART model selected only five predictors, as is shown in Figure 2. The first split was made on the Database score: if the probes in the candidate segment were included in the DGV at least 2.45 times on average, the candidate was predicted to be a CNV. Otherwise, only segments with the following characteristics were predicted to be CNVs: 1) segments shorter than 30 Kb; or 2) segments of length longer than 30 Kb that had matching candidate segments in 37% or more of the other patients. The prediction accuracy of this model, estimated for the smoothed CBS test set, was 86%, as shown in the Table 3. Table 4 has the numbers of candidate segments that were correctly and falsely classified. There were 793 true CNVs predicted to be CNVs, and 654 correctly identified true CNAs. Interestingly, frequency of CNAs falsely identified as CNVs (182) was much higher than frequency of missed CNVs (54). We believe CNVs were easier to identify because the DGV contains a lot of information about them.

The other two validation sets we were using were the set of 39 tumors that were hybridized against self-reference, thus, all its 1780 candidates were true CNAs, and the set of 8 normal tissue arrays containing 257 true CNVs only. As seen in Table 3, 79% and 90% of these segments, respectively, were identified correctly. As in the test set, the rate of missed CNVs was smaller.

The RF model with the same set of predictors increased the accuracy by 1% on the test set, and by 3-5% on the 'all CNAs' and 'all CNVs' sets. Since the best model was a combination of many trees it is difficult to present it visually; however, relative importance of each variable measured by Gini index is shown in Table 5. The more influential variables have higher indices. The ranking of the variables was roughly consistent with univariate results: the top predictors were Database score, Length, Matching breakpoint in other patients - percent, Overlap with other patients - percent, Percent of Normal and Relative height.

We have also fitted the CART model with a single variable - Database score. Its only split was the same as the first split of the full model: segments seen in the DGV on average 2.45 times were predicted to be CNVs. The prediction accuracy of this model was equal to 85%, 88% and 81% on the test, 'all CNAs' and 'all CNVs' sets respectively. Therefore, using all the proposed predictors on the test set in addition to the Database score increased the accuracy on the test set by 2%.

The fitted CART tree was different in GLAD segmentation, although the first split was still made on the Database score. As seen in panel (b) of Figure 2, segments were predicted to be CNVs if 1) they were included in the DGV at least 3 times on average and had relative absolute mean greater than 1.5; or 2) they were included in the DGV less than 3 times on average and overlapped with other candidates in at least 38% of other patients of the cohort. In the RF model for GLAD segmentation, the 6 variables with the highest Gini
indices (Table 5) were the same as in smoothed CBS segmentation, while having slightly different ranking. In spite of these differences, the prediction characteristics of models based on GLAD and smoothed CBS were similar. The RF with all predictors correctly identified 86% of candidates in the test set, 91% of 1861 true CNAs in ‘all CNA’ dataset, and 87% of 247 true CNVs in ‘all CNVs’ set, while the rates of false CNVs and false CNAs were more balanced. The model with only Database score had the same first split of Database score greater or less than 3, and its accuracy on the test set was only 3% smaller than that for the full model.

Since many of the predictors were highly correlated there could be many classification trees with similar prediction accuracy, so the difference in models between GLAD and smoothed CBS might be a result of random variation rather than fundamental segmentation differences. In fact, when we applied the full RF developed on the GLAD segmented training set to smoothed CBS test set, the prediction accuracy was 87%, which was the same as the model developed based on smoothed CBS. Similarly, the RF developed on the smoothed CBS training set resulted in 83% accuracy as assessed in GLAD test set, just 3% smaller than the RF developed on the GLAD training set.

Prediction modeling based on unsmoothed CBS had several important differences. The Variable Matching breakpoint in other patients - percent served as a first split in the classification tree. If a segment 1) had candidates with matching breakpoints in more than 1.2% of other patients and was either shorter than 396 Kb or was both longer than 396 Kb and was included in the DGV on average 4.5 times; or 2) had candidates with matching breakpoints in less than 1.2% of other patients, was shorter than 22 Kb, or shorter than 77 Kb and included in the DGV on average 1.3 times, or longer than 77 Kb and included in the DGV on average 3.1 times, then it was predicted to be a CNV. Note that in our training set the first split is equivalent to having at least one other tumor with exactly matching breakpoint of a candidate. RF had the same 6 variables with the highest Gini indices as two other segmentation methods, and it correctly predicted 84% of segments in the test set, as well as 77% of 1785 CNAs and 92% of 464 CNVs in the two other validation sets. Unlike in smoothed CBS and GLAD, the classification tree that included only Database score showed only 72% accuracy on the test set, 12% lower than the full model. This model had a much higher false CNV rate - 66% of all CNAs in the test set and 66% of the CNAs in ‘all CNAs’ validation set were falsely identified as CNVs (see Tables 3, 4). We speculate that unsmoothed CBS contained smaller intervals that rarely appeared in the DGV, and the Database score was less informative about them. As a result the model had lower prediction rates on the validation sets.

While the DGV provided the strongest univariate information, we investigated whether it was absolutely necessary for predicting CNVs by fitting RF that excluded Database score and Database score of other candidates. We saw only a modest drop in prediction accuracy of 0-2%. The most important variables suggested by the Gini index Matching breakpoint in other patients - percent, Overlap with other patients - percent, Length, Relative height, and Percent of Normal were the same across all three segmentation methods.

We have also considered models that excluded Overlap with other patients and Overlap...
Table 3: Prediction rates: A - test set, B - CGH against self-reference (all CNAs), C - normal tissue (all CNVs)

<table>
<thead>
<tr>
<th></th>
<th>CBS smoothed</th>
<th>GLAD</th>
<th>CBS unsmoothed</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>B</td>
<td>C</td>
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<td>0.90</td>
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<td>RandomForest - no database</td>
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<tr>
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<td>0.97</td>
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Table 4: Counts of prediction on the test set. (TN, True Negatives, are true CNAs predicted to be CNAs; FN, False Negatives, are true CNVs predicted to be CNAs; FP, False Positives, are true CNAs predicted to be CNVs; TP, True Positives, are true CNVs predicted to be CNVs)

<table>
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<th>GLAD</th>
<th>CBS unsmoothed</th>
</tr>
</thead>
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with CNAs, and thus could be applied to a single array. They are presented in the last row of Tables 3 and 4. There was 1-2% loss of accuracy compared to the full models.

All the analyses were performed in R (http://www.r-project.org/) and the final RF models are collected in a .RData file, which is available online along with a short manual.

4 Discussion

In this article we introduced a framework for distinguishing germline copy number variants (CNVs) from cancer-related copy number alterations(CNAs) when analyzing tumor samples on copy number arrays. To our knowledge, our manuscript is the first attempt to quantify the overlap of a given copy number abnormality with the database of genomic variants (DGV) and to suggest a rule for determining CNVs. We have also examined various characteristics of the altered segments that can be different between CNVs and CNAs.

We considered three segmentation methods to identify candidate CNVs and built CART and RF prediction models using up to 16 predictors that can be applied to both cohorts of several independent patients and to single arrays. If the segmentation was done after removing outliers then the most important predictor was overlap with DGV. If each probe of a candidate segment overlapped on average with 2.5 - 3 variants listed in the DGV, this candidate segment was likely to be a CNV. Inclusion of additional variables like Length,
Table 5: Relative importance of variables in random forest models as measured by Gini index (higher is more important). "'W. DS'" stands for the model that includes Database score (DS), "'w/o DS'" stands for the model where it was excluded.

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<td>w. DS</td>
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Figure 2: Fitted CART models
Relative height, Overlap with other patients in the cohort improved the accuracy by a few percent. The model developed using one segmentation method can be successfully applied to another equivalent segmentation (smoothed CBS and GLAD). The advantage of additional predictors was more pronounced (12% higher accuracy) if the segmentation was performed on data with no outliers removed. Such data were more likely to contain smaller candidate segments that are missed in the DGV.

Overall, the prediction accuracy in the test set is around 85% across different segmentation methods. We have also applied the classification algorithm to validation sets containing only CNVs (normal samples) or only CNAs (tumor samples with CNVs subtracted as a reference). The candidate segments were correctly classified in these datasets in 80-95% of cases, even though our classification model was not developed on these types of samples.

Note that the variable Database score, while being one of the most significant, is based on the DGV, which has some inaccuracies and repetitions. For each probe it involves the count of variants listed in the DGV that covered that probe. These variants, however, are not independent and representation of many of them in the DGV is redundant. For example, many studies report identical or almost identical variants observed on the same sets of patients (e.g. HapMap patients), and few variants are listed twice even within the same study. In addition, variants have been observed in a different number of people and have different frequencies, although our variable Database score II that utilized these frequencies did not prove to be superior, probably also due to redundancy in variant reporting. Note that CNVs discovered by fine scale mapping of DNA from HapMap patients (Perry et al., 2008) were much smaller, often by more than 50%, than CNVs reported in the DGV based on previous studies. As the DGV and the frequencies of all known CNVs become more accurate the prediction model can be improved.

Interestingly, when we have used a previous version of the DGV that contained about half of the variants listed in March 2009, our prediction model with Database score only was only about 3% less accurate. That might mean that either the new studies find areas of CNVs that are known already, or variability of CNVs in the population is so large that expansion of DGV does not help to predict CNVs in a randomly chosen patient. The Database score and Overlap with other patients are still the most significant variables across segmentation methods, and no other characteristic of a single segment is a strong predictor of CNV, so analyzing genomes from different people and populations is still the best way to increase our knowledge about CNVs.

Although we have identified true CNVs by matching the candidate segments in tumors to their corresponding normal samples, our classification is not a gold standard. Due to segmentation error and possibly imperfect gain/loss calling, some CNVs and CNAs in the tumors might have been missed. It is also plausible that some true CNVs were missed by our matching method and classified as true CNAs. However, since we have verified the prediction framework on several validation sets, we do not expect the error in true CNV classification to have had a strong impact on the model. We also believe that the fitted models are not specific to glioblastoma since CNVs should be mostly homogeneous across patients with different cancers, and the good predictive ability even on the set of normal
samples supports this claim.

Our models do not depend on the scale of log-ratios since the only important predictor that depends on them, absolute segment mean, is divided by the median absolute deviation of noise and as a result, Relative height is scale and noise level invariant. Nevertheless, all our analyses are done on Agilent 244K arrays, and CNVs might have different characteristics if they were detected on arrays of different resolution or different platforms. We believe that the model will be as efficient on arrays that have similar or worse quality and resolution, since they likely identify CNVs that are similar to or a subset of what can be found by the Agilent 244K platform.

The Agilent 244K array, like all non-SNP arrays, measures total copy number rather than allele-specific copy number. That is, it cannot separately estimate the two parental copy number contributions. This could lead to occasional error in our analysis. For instance, if there were CNVs on both alleles, and if there were a copy neutral LOH event (one parental copy number doubles while the other disappears) that was larger than the LOH event, it is possible that we would interpret this event as a CNA (which would be correct), a CNV, or a normal region. The interpretation would depend on the allelic copy numbers of both the normal and tumor samples. This problem, however, is due to the limitation of the array, not to our algorithm.

Many studies that have the goal of identifying cancer genes deliberately exclude CNVs prior to analysis (e.g. TCGA-Network, 2008). One way to do this using our algorithm would be to segment the original data and apply the appropriate RF model. The probes within predicted CNVs in at least one, or, conservatively, several patients could be excluded, and the reduced data set could be segmented again for the final analyses. Alternatively, since predictions are obtained for all segments that are located in areas of suspected recurrent gain or loss, a region might be discarded if some or many of the matching candidates are predicted to be CNVs.

It is also interesting to study CNVs in cancer patients. For example, there is evidence that CNVs may contribute to chromosome breakage (Camps et al., 2008) and to cancer risk (Liu et al., 2009, Diskin et al., 2009). There are abundant studies of copy number on cancer patients that are publicly available. For any of these studies the data can be segmented and all the candidate segments can be classified as CNVs or CNAs. The presence of CNVs as identified by the proposed method can be correlated with recurrent CNAs or clinical characteristics. Therefore, the classification model that we developed may facilitate the study of the associations between CNVs and cancer predisposition or progression.

### 4.1 Acknowledgments

We thank Cameron Brennan and Marc Ladanyi for helpful comments. The results published here are in whole or part based upon data generated by The Cancer Genome Atlas pilot project established by the NCI and NHGRI. Information about TCGA and the investigators and institutions who constitute the TCGA research network can be found at http://cancergenome.nih.gov. This research was supported in part by NIH U24 grant CA126543 (The Cancer Genome Atlas) (to A.O.)
References


## 5 Appendix
Table 6: Excluded physiological regions

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Table 7: Regions of segmental duplication (hg18)

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