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Kinetochore genes are coordinately up-regulated in human tumors as part of a FoxM1-related cell division program

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ABSTRACT The key player in directing proper chromosome segregation is the macromolecular kinetochore complex, which mediates DNA-microtubule interactions. Previous studies testing individual kinetochore genes documented examples of their overexpression in tumors relative to normal tissue, leading to proposals that up-regulation of specific kinetochore genes may promote tumor progression. However, kinetochore components do not function in isolation, and previous studies did not comprehensively compare the expression behavior of kinetochore components. Here we analyze the expression behavior of the full range of human kinetochore components in diverse published expression compendia, including normal tissues and tumor samples. Our results demonstrate that kinetochore genes are rarely overexpressed individually. Instead, we find that core kinetochore genes are coordinately regulated with other cell division genes under virtually all conditions. This expression pattern is strongly correlated with the expression of the forkhead transcription factor FoxM1, which binds to the majority of cell division promoters. These observations suggest that kinetochore gene up-regulation in cancer reflects a general activation of the cell division program and that altered expression of individual kinetochore genes is unlikely to play a causal role in tumorigenesis.

INTRODUCTION Proper chromosome segregation during mitosis is central to maintaining genome integrity. Even subtle chromosome segregation defects result in inappropriate chromosome numbers (termed aneuploidy), which are observed in >70% of tumors and have been suggested to promote tumorigenesis (Holland and Cleveland, 2009). Thus it is critical to understand the perturba-

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(Takimoto et al., 2002), CENP-A (Tomonaga et al., 2003), CENP-H (Tomonaga et al., 2005; Shigeishi et al., 2006), and many others.

The observations that selected kinetochore genes are up-regulated in tumors has led to proposals that this up-regulation may act as a causal event for tumor initiation or progression (Yuen et al., 2005). In a subset of cases, this hypothesis has been tested using mouse models in which the expression of a given kinetochore gene is artificially up-regulated. Strikingly, up-regulation of Mad2 (Sotillo et al., 2007), Ndc80/Hec1 (Diaz-Rodriguez et al., 2008), or Aurora B (Ricke et al., 2011) can increase the incidence of cancer formation in mice. Although these studies demonstrate that up-regulation of a kinetochore gene has the potential to promote tumorigenesis, the physiological relevance of these up-regulation events in human tumors remains unclear.

Here we seek to evaluate comprehensively kinetochore gene expression in diverse, publicly available data sets to determine the relative expression behavior of kinetochore genes. Our results demonstrate that core kinetochore genes are coordinately expressed under all analyzed conditions, including in normal human tissues, tumors, and cancer cell lines. In addition, kinetochore genes show similar expression behavior to other cell division genes. Most kinetochore genes do not display strong periodic mitosis-specific expression during the cell cycle, indicating that the observed up-regulation is not due solely to an increased proportion of mitotic cells in tumors. Instead, this expression behavior appears to reflect the induction of a broad but specific cell division program. To define key upstream factors involved in the induction of this cell division program, we analyze the relationship between kinetochore gene expression and established transcription factors. Our data suggest that this expression program is closely related to the behavior of the forkhead-family transcription factor, FoxM1. Thus kinetochore genes in tumors are coordinately expressed as part of a general activation of the cell division program that appears to function downstream of FoxM1.

**RESULTS**

**Selection of kinetochore and cell division genes for expression analysis**

To analyze the relative expression of kinetochore genes, we first generated a list of genes that could be used to assess expression profiles across diverse cell types. Work from our laboratory and many others has identified >100 different kinetochore-localized proteins in human cells (Cheeseman and Desai, 2008). These kinetochore proteins can be grouped into two broad functional categories: 1) core kinetochore proteins, which function exclusively at kinetochores; and 2) multifunctional kinetochore proteins, which function at kinetochores and additional subcellular locations (e.g., the microtubule-based motor cytoplasmic dynein or the nucleoporin Nup107-160 complex). To analyze the expression of other key genes required for dividing cells, we also selected genes involved in DNA replication and cell cycle regulation. Finally, to provide a comparison to both normal cellular processes and cancer states, we selected several representative housekeeping genes and genes whose up-regulation has been implicated in tumor progression (e.g., Myc and Ras). In total, we defined five functional categories that we refer to as follows: Core Kinetochore, Multifunctional Kinetochore, Cell Cycle/DNA Replication, Housekeeping, and Cancer Up-regulated. The selected genes and the corresponding categories are given in Supplemental Table S1.

**Kinetochore genes display coordinated expression in normal tissues**

We first assessed the expression of our selected genes in normal tissue samples using data from the Human Body Index (HBI; GEO Accession No. GSE7307; www.ncbi.nlm.nih.gov/geo). We normalized the expression of each gene relative to its median expression level across the entire set of samples and plotted these data as a heat map to indicate the level of up-regulation or down-regulation relative to this median expression (Figure 1). In this analysis, the mRNA levels of the Core Kinetochore genes were positively correlated (correlation of 0.44), representing coordinate expression across the entire selection of tissue samples. The highest levels of expression for these genes were observed in testes, bone marrow, and other tissues believed to show higher overall levels of cell division. Core Kinetochore components were expressed at lower levels in terminally differentiated tissues, including brain and neuronal samples. In this analysis, our selected Cell Cycle/DNA Replication genes displayed similar expression profiles to Core Kinetochore genes across these diverse samples. In contrast, as expected based on the role of the cytoplasmic dynein motor in axonal transport, we found that some Multifunctional Kinetochore genes, including dynein and its multiple associated proteins, were up-regulated in brain and neuronal samples. On the basis of the multiple cellular functions for these Multifunctional Kinetochore proteins, as well as their distinct and diverse behaviors in the data sets analyzed later (unpublished data), for simplicity we excluded these genes from subsequent figures. This analysis indicates that the selected Core Kinetochore and cell division genes are coordinately expressed across noncancerous human tissue samples.

**Cell cycle expression of kinetochore genes**

In the human tissue samples analyzed, Core Kinetochore gene expression appeared to correlate with the presumptive cell division frequency (mitotic index) of a given tissue. If these genes are up-regulated during the mitotic phase of the cell cycle, the increased expression observed in these samples could be due to an increased proportion of mitotic cells. Similarly, if Cell Cycle genes are expressed in a specific cell cycle stage, their increased expression in frequently dividing tissues could be due to increased occupancy of that cell cycle stage. Therefore we sought to assess the relationship between the expression of these genes and cell cycle stage. To test the relationship between Core Kinetochore gene expression and cell cycle progression, we analyzed periodic gene expression in a data set that analyzed time points in synchronously dividing human cells (Grant et al., 2013). We found that a subset of Core Kinetochore genes was periodically expressed during the cell cycle (Figure 2). These periodically expressed genes are enriched for outer kinetochore proteins and regulatory components that localize to kinetochores only during mitosis. However, the majority of Core Kinetochore genes and other cell division components did not show dramatic differences in their expression behavior during the cell cycle (Figure 2). This suggests that the up-regulation of Core Kinetochore and Cell Cycle genes observed in frequently dividing normal tissues represents a general activation of the cell division program rather than expression due to increased occupancy of a specific cell cycle stage.

**Kinetochore genes are up-regulated in tumors**

Multiple studies demonstrated that individual kinetochore genes are up-regulated in tumors relative to normal tissue controls (see Introduction). To test whether this up-regulation is broadly true for other Core Kinetochore and Cell Cycle/DNA Replication
components, we analyzed their gene expression in The Cancer Genome Atlas (TCGA) study of breast cancer (Cancer Genome Atlas Network, 2012), in which matched breast tumor samples and normal tissue controls were analyzed for 28 different patients (Figure 3). For this analysis, gene expression in each individual tumor sample was normalized relative to the level of gene expression in the corresponding matched normal tissue sample. In this analysis, half of the Core Kinetochore and Cell Cycle/DNA Replication genes (36 of 71 genes) were significantly up-regulated (false discovery rate \( p < 0.05 \) and at least twofold change, t test) in the tumor samples relative to the normal tissue controls. These data suggest that tumors broadly activate genes required for cell division rather than up-regulate individual cell division genes.

**Kinetochore genes are coordinately regulated in diverse tumor and cancer samples**

The matched tumor/normal tissue samples provide an important comparison for evaluating the up-regulation of kinetochore genes. However, these paired experiments are of limited sample size, preventing an extensive analysis of the coordinated behavior of kinetochore genes across different conditions. Therefore we analyzed the relative expression of our selected genes in the Broad Cancer Cell Line Encyclopedia (Figure 4), which contains expression data for 991 cancer cell lines (www.broadinstitute.org/ccle; Barretina et al., 2012), and the Expression Project for Oncology (expO) data set (Figure 5), which contains 2158 tumor samples (GEO Accession No. GSE2109). For these analyses, we first defined the median expression level for a given gene across all of the samples in a data set. We then compared its expression level in each individual sample to that median expression level to define the relative level of up- or down-regulation. This normalized expression level for a given gene across the diverse samples allowed us to evaluate the variation in gene expression among tumor populations. Based on this methodology, half of the samples should display reduced expression relative to this median level. However, based on the analysis of the matched TCGA breast cancer samples (Figure 3), each kinetochore gene is likely to be up-regulated relative to normal tissue.

Our analysis of gene expression behavior in tumor and cancer samples demonstrated that there is a strong similarity between changes in the mRNA levels of the Core Kinetochore components and Cell Cycle/DNA Replication genes (Figures 4 and 5). This expression profile was distinct from the other classes of genes.
expression of Mad1 (Mad1L1) and p31/Comet (Mad2L1BP) in skin cancer cell lines from the CCLE data set (Figure 4). Therefore most individual kinetochore genes are not up-regulated in isolation, but instead their expression represents the broad induction of Core Kinetochore and Cell Cycle genes.

Although we found that Core Kinetochore genes generally behaved similarly to each other, as a group they exhibited variation in mRNA levels across the different tumors and cancer cell lines.
We therefore sought to determine whether this relative expression behavior correlated with different tumor properties or behaviors. We did not detect an obvious correlation between the level of Core Kinetochore gene expression and the pathological stage of the tumor in the expO tumor data (unpublished data). Similarly, we did not observe any noticeable difference in the expression behavior in those tumors in the expO data set from patients who smoked relative to nonsmokers (unpublished data). In addition, we did not detect differences in the sensitivity of the cell lines from the CCLE data set to antimitotic drugs, including Taxol, based on the varying Core Kinetochore gene expression levels (unpublished data).

FIGURE 3: Kinetochore gene expression in tumors vs. normal tissue. Gene expression data were obtained from the TCGA study of invasive breast cancer (Cancer Genome Atlas Network, 2012). Expression levels of the selected genes in tumor samples were compared with the expression levels in the corresponding matched normal tissue control, with changes in expression level color coded as indicated. To the left of the expression data, a hierarchical tree indicates the relationship between expression patterns. To the right of the expression data, genes are classified based on their functional category.
In contrast, we observed a clear relationship between the mRNA level and the identity of the tissue from which the tumor or cell line was derived. For example, much higher levels of Core Kinetochore and Cell Cycle/DNA Replication genes were observed in colon, cervix, and uterus samples, with lower relative levels present in kidney, prostate, and thyroid samples (Figures 4 and 5). Because matched normal/tumor samples are not available in the expO data set, we compared the expO tumor expression data to the tissue-specific expression levels from the HBI. Based on this comparison, the expression of Core Kinetochore genes is up-regulated to a roughly similar extent in these diverse tumor samples relative to the corresponding tissue of origin (Figure 6). Thus these differing expression levels primarily reflect the basal state of the starting tissue rather than a specific feature of tumorigenesis in a given tissue.
observed highly correlated expression profiles for kinetochore genes in the CCLE cancer cell line data and expO tumor data set (Figure 7).

We also sought to analyze the expression of specific kinetochore protein complexes for which up-regulation in cancers has been reported previously. Ndc80/Hec1 assembles as part of a four-subunit Ndc80 complex that includes Nuf2, Spc24, and Spc25. Ndc80/Hec1 has been shown to be up-regulated in tumors (Chen et al., 1997; Hayama et al., 2006), and mice overexpressing Ndc80 display increased cancer formation (Diaz-Rodriguez et al., 2008). If the Ndc80

**Kinetochoore protein interaction partners display closely coordinated expression**

The similar expression behavior observed in tumors and cancer cell lines for the Core Kinetochore genes has important implications for the consequences of this altered expression. Because most kinetochoore proteins exist in multisubunit protein complexes, the relative protein levels of the different subunits, rather than the precise amounts of a given subunit, will affect the behavior of that complex. Therefore we sought to analyze the statistical correlation for the gene expression behaviors of Core Kinetochore genes. Overall we...
FIGURE 6: Tumor gene expression normalized relative to corresponding normal tissue. Gene expression data from the expO tumor data set were compared with the median expression in the corresponding normal tissue from the HBI data set. For example, kidney tumors were compared with the median expression in normal kidney tissues. These data suggest that tumors display similar degrees of up-regulated expression of kinetochore genes. To the left of the expression data, a hierarchical tree indicates the relationship between expression patterns. To the right of the expression data, genes are classified based on their functional category.
protein were specifically up-regulated in the absence of its binding partners, this would cause severe defects with the potential for dominant-negative consequences to the fidelity of chromosome segregation. In contrast, coordinate mRNA up-regulation of the Ndc80 complex subunits would avoid defects due to unbalanced subunit components. On the basis of our analysis, we found similar expression behavior for Ndc80, Nuf2, and Spc25 in cancer cell lines and tumors (correlation of 0.64 in CCLE data and 0.78 in expO data; correlation of 0.43 in the CCLE data set and 0.85 in the expO data sets). However, previous work on the Mis12 complex found that the protein levels of the Mis12 complex subunits are related as the Ndc80 complex (0.26 in the CCLE data set and 0.16 in the expO data sets). Therefore, the level of Mad2 alone occurs in human tumors. Cells are exquisitely sensitive to the level of Mad2, with subtle changes in concentration altering spindle assembly checkpoint signaling and mitotic progression (Heinrich et al., 2013). However, the absolute level of Mad2 in a cell does not appear to be the primary factor controlling checkpoint function. Instead, the level of Mad2 relative to its binding partner and downstream target Cdc20 is the key, determining factor (Heinrich et al., 2013). Our analysis of the tumor and cell line expression data revealed that Mad2 and Cdc20 displayed highly coordinated gene expression across the diverse samples (correlation of 0.43 in the CCLE data and 0.85 in the expO data).

Finally, we tested the correlation between the subunits of the Mis12 complex (Mis12, Dsn1, Nnf1/Pmf1, and Nsl1) form an integrally associated, stable subcomplex (Kline et al., 2006) but have not been a focus of previous studies on kinetochoore gene up-regulation in cancer. Although Mis12 complex subunits show similar expression to other kinetochoore genes in our analyses, the four subunits of this complex are not as strongly correlated as the Ndc80 complex (0.26 in the CCLE data set and 0.16 in the expO data sets). However, previous work on the Mis12 complex found that the protein levels of the Mis12 complex subunits are very sensitive to each other. For example, Dsn1 appears to be very unstable in the absence of the other subunits, both in cells (Kline et al., 2006) and in biochemical reconstitution experiments (Cheeseman et al., 2006). Although we focused here on the transcriptional control of kinetochoore gene expression, translational and posttranslational control of protein levels will also affect the final subunit balances present in the cell.

![Cancer Cell Line (CCLE) Gene Correlations](image1)

![expO Gene Correlations](image2)

**FIGURE 7:** Correlations for kinetochoore gene expression data from the CCLE and expO data sets. To assess the correlations in the gene expression profiles of the kinetochoore genes (as well as the FoxM1 and E2F1 transcription factors), pairwise comparisons between the expression profiles for each pair of genes were tested in the CCLE and expO data sets. To the left of the expression data, a hierarchical tree indicates the relationship between gene correlations. Overall kinetochoore genes display highly correlated gene expression profiles in tumors and cancer cell lines.
Overall, our analyses indicate that despite previous reports, Mad2 and Ndc80/Hec1 do not appear to be specifically up-regulated in cancer and tumor samples and instead are expressed as part of a larger cluster containing the Core Kinetochore and Cell Cycle/DNA Replication genes.

Expression of the FoxM1 transcription factor is highly correlated with expression of cell division genes

The coordinated expression of the Core Kinetochore and Cell Cycle/DNA Replication genes across the diverse data sets that we analyzed suggests that there is a transcriptional basis for this similar expression. To evaluate this, we analyzed the expression of genes that have been shown to control key gene expression programs, including the forkhead-type transcription factors and the proliferation-promoting transcription factors E2F1 and E2F4.

E2F1 displayed moderately correlated gene expression to Core Kinetochore components in the CCLE and expO tumor data sets (correlations of 0.38 and 0.26, respectively). However, the E2F4 transcription factor did not show similar expression to kinetochore components (correlation $\sim 0.09$). This is consistent with our observations that the expression behavior of the cell division genes is not strictly related to increased expression of periodically expressed genes, many of which are regulated downstream of E2F (Ren et al., 2002).

The majority of forkhead-family transcription factors (including FoxA1, FoxC2, FoxO1, FoxO3, and FoxP3) did not display detectable similarity to the expression of the Core Kinetochore genes and Cell Cycle/DNA Replication components. In contrast, we found that FoxM1 showed highly correlated expression to kinetochore components in the expO tumor data (Figure 7; correlation 0.65). FoxM1 binds to promoter regions of the majority of kinetochore genes and cell division components based on recent chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-seq) analyses (Figure 5; ENCODE Project Consortium et al., 2012; Chen et al., 2013; Grant et al., 2013; Sanders et al., 2013). In particular, FoxM1 displays at least 10-fold enrichment of its ChIP-seq signal compared with a control IP in a region encompassing 1 kb upstream and 100 base pairs downstream of the transcriptional start site for 48 of 56 Core Kinetochore genes. This suggests that FoxM1 contributes to the coordinated expression of Core Kinetochore and Cell Cycle genes observed in normal tissues and cancer samples. Indeed, previous work found that FoxM1 is required for cell cycle progression and expression of multiple cell division genes (Laoukili et al., 2005; Grant et al., 2013). Coordinated expression of FoxM1-dependent genes suggests that regulation of FoxM1 itself could be a fundamental step in activating the cell division expression program in normal tissues and cancers.

DISCUSSION

Previous work documented numerous cases in which a given kinetochore gene is overexpressed in a selection of tumors. These observations led to proposals that such overexpression could be a causal event in promoting tumorigenesis (Yuen et al., 2005). In fact, recent work shows that artificially up-regulating individual kinetochore genes can promote tumor formation in mice (Sotillo et al., 2007; Diaz-Rodriguez et al., 2008; Ricke et al., 2011), but a broad-based consideration of whether such up-regulation of individual genes occurs in human tumors has been lacking.

To evaluate the significance of observed gene up-regulation in cancer, defining the basis for this up-regulation is of critical importance. There are several mechanisms by which a gene could be up-regulated. First, the expression of its corresponding upstream transcription factors could be changed. In this case, the altered expression of a transcription factor would be likely to affect the behavior of multiple downstream genes. Second, a promoter or enhancer region of an individual gene could be altered to increase the level of transcription. Third, the copy number of the gene could be increased through amplification or other genomic changes. However, because large genomic regions are typically amplified in cancers, it can be hard to assess which individual gene(s) within this amplified region are responsible for tumor behavior.

In cases in which a kinetochore gene is amplified or its promoter region is altered, this would be predicted to affect the mRNA level of that gene but not other kinetochore components. Such cases could represent causal events in tumor progression, as they could disrupt subunit balances within the macromolecular kinetochore structure and result in dominant-negative defects in kinetochore function, compromising the fidelity of chromosome segregation. However, it was unclear whether up-regulation of individual kinetochore genes occurs in human tumors. Here we analyzed kinetochore gene expression in diverse, publicly available expression compendia, which revealed that kinetochore genes are largely coordinately expressed across diverse conditions. On the basis of this strongly correlated behavior, we conclude that the up-regulation of an individual kinetochore gene is unlikely to be a causal factor in the vast majority of human cancers. However, we cannot exclude the possibility that minor changes in gene expression (less than twofold) do occur for some kinetochore components. Based on recent analysis in fission yeast (Heinrich et al., 2013), such subtle imbalances for some kinetochore components could result in changes in mitotic progression or fidelity. We note that detecting such subtle changes in expression would be beyond the sensitivity of most experimental approaches, and experiments testing the consequences of artificially up-regulating kinetochore genes have induced >10-fold overexpression of a selected component.

Instead of specifically up-regulating specific kinetochore components, cancers appear to activate a broad-based cell division program that involves both the core kinetochore components and other genes required for cell cycle progression and DNA replication. We propose that this is not strictly related to mitotic index or doubling time of the cancer cells. Indeed, most kinetochore genes are not strongly periodically expressed during the cell cycle. Instead, this appears to be a general activation of the cell division program. Previous work found similar gene clusters for genes that function in related processes, such as ribosomal or proteasomal components (Niehrs and Pollet, 1999). On the basis of the observed coexpression behavior of the FoxM1 transcription factor and the binding of FoxM1 to the promoters of cell division genes, we propose that the up-regulation of the cell division program occurs downstream of FoxM1. Activation of this cell division program may allow a cell to be poised for cell division such that these proteins are ready to facilitate mitosis once additional cues signal cell cycle entry.

Our analyses provide an important context for considering the roles of kinetochore components in driving tumor progression and highlight the general activation of the cell division program in tumors. Because many cancer cells have been shown to display error-prone chromosome segregation (chromosomal instability; Thompson et al., 2010), it is important to define the molecular basis for this behavior. Our analysis suggests that dramatic changes in the expression of an individual kinetochore gene are unlikely to be responsible for chromosome instability. In contrast, the recent sequencing of cancer cell (Barretina et al., 2012) genomes revealed widespread mutations in components of the kinetochore and cell division apparatus that represent interesting candidates for future analyses.
For TCGA breast invasive carcinoma data, processed Agilent expression data (level 3) for matching normal and tumor pairs (sample IDs are given in Table 1) were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov). For each cancer sample, log2 ratios of selected genes were calculated relative to the matching normal sample, and the heatmap was created as described.

A subset of the expO tumor samples was also compared with normal counterparts in the HBI compendium. For each of the common HBI tissues (kidney, colon, liver, breast, lung, ovary/uterus, and prostate), the median expression level of each gene in that tissue was calculated, and each expO tumor sample was compared with the set of corresponding normal medians. The heatmap was generated as described.

Pearson correlations were calculated in R by comparing the expression (log2 ratios) of each gene against every other gene in the data set. The correlation values were then clustered in Cluster3 and visualized in Java TreeView, as described. Correlations were summarized by the mean via Fisher transformation.

FOXM1 ChIP-Seq peaks were downloaded from the ENCODE project (https://genome.ucsc.edu/ENCODE/downloads.html, under MATERIALS AND METHODS

Data processing and analysis

For each expression compendium (HBI [GEO Accession No. GSE7307], expO [GEO Accession No. GSE2109], and CCLE [www.broadinstitute.org/ccle]), Affymetrix Human Genome U133 Plus 2.0 arrays were normalized with robust multiarray average (RMA) using the affy package from Bioconductor (www.bioconductor.org/packages/release/bioc/html/affy.html) and Entrez Gene (www.ncbi.nlm.nih.gov/gene) ID custom probeset definitions as defined previously (Dai et al., 2005). The RMA values of selected genes were subsequently median centered and hierarchically clustered (based on uncentered correlation and average linkage) using Cluster 3.0 (de Hoon et al., 2004) and visualized with Java TreeView (Saldanha, 2004). Principal component analysis plots gave similar results to the hierarchical clustering (unpublished data).

For the cell cycle study (Grant et al., 2013), processed Agilent Whole Human Genome Oligonucleotide array ratios (sample/reference) were downloaded from Suplemental Table S1. The profiles of selected genes were sorted by arctan2 values, as provided in the table.

TABLE 1: TCGA samples.

<table>
<thead>
<tr>
<th>TCGA tumor samples</th>
<th>TCGA normal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCGA-BH-A0AU-01A-11R-A12P-07</td>
<td>TCGA-BH-A0AU-11A-11R-A12P-07</td>
</tr>
<tr>
<td>TCGA-BH-A0BS-01A-11R-A12P-07</td>
<td>TCGA-BH-A0BS-11A-23R-A12P-07</td>
</tr>
<tr>
<td>TCGA-BH-A0BS-01A-11R-A12P-07</td>
<td>TCGA-BH-A0BS-11A-23R-A12P-07</td>
</tr>
<tr>
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<td>TCGA-BH-A18Q-11A-34R-A12D-07</td>
</tr>
</tbody>
</table>

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