Motivation:
Alternative splicing is central for cellular processes and substantially increases transcriptome and proteome diversity. Aberrant splicing events often have pathological consequences and are associated with various diseases and cancer types. The emergence of next generation RNA sequencing (RNA-seq) provides an exciting new technology to analyse alternative splicing on a large scale. However, algorithms that enable the analysis of alternative splicing from short-read sequencing are not fully established yet and there are still no standard solutions available for a variety of data analysis tasks.

Results:
We present a new method and software to predict genes that are differentially spliced between two different conditions using RNA-seq data. Our method employs geometric angles between the high dimensional vectors of exon read counts. With this, differential splicing can be detected even if the splicing events comprise of higher complexity and involve previously unknown splicing patterns. We applied our approach to two case studies including neuroblastoma tumour data with favourable and unfavourable clinical courses. We show the validity of our predictions as well as the applicability of our method in the context of patient clustering. We verified our predictions by several methods including simulated experiments and complementary in silico analyses of the biological data itself. We found a significant number of exons with specific regulatory splicing factor motifs for predicted genes and a substantial number of publications linking those genes to alternative splicing. Furthermore, we could successfully exploit splicing information to cluster tissues and patients. Finally, we found additional evidence of splicing diversity for many predicted genes in normalised read coverage plots and in reads that span exon-exon junctions.

Availability:
SplicingCompass is licensed under the GNU GPL and freely available as a package in the statistical language R at http://www.ichip.de/software/SplicingCompass.html
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1 INTRODUCTION

Alternative splicing is a central cellular process that produces different mRNA isoforms from a single gene. The qualitative and quantitative identification of such isoforms is essential for understanding the different roles of alternatively spliced genes in a cell. In addition, the detection of disease-specific isoforms is an important task since aberrant splicing is known to be responsible for various diseases (reviewed in Kim et al. (2008a)) and associated with different cancer types (e.g. Christofk et al. (2008); Venables et al. (2009)).

Isoform structures and abundances can be inferred from short sequence reads generated by RNA-seq. In contrast to hybridisation based approaches using e.g. microarrays, such sequencing based approaches allow the detection of previously unknown splicing events and can avoid cross-hybridisation and limited dynamic range (reviewed in Wang et al. (2009)). Computational methods for analysing differential expression and differential splicing from RNA-seq data “are only beginning to emerge” (reviewed in Garber et al. (2011)). Existing methods detect alternative splicing mainly by considering sequencing reads that map uniquely to single isoforms or by assembling transcripts and estimating the most likely isoform abundance levels according to the given sequencing reads (an overview and discussion of limitations is given in Martin and Wang (2011) and Garber et al. (2011)). Short-read assemblers have been developed for genome and transcriptome assembly (e.g. Velvet (Zerbino and Birney, 2008), Scripture (Guttman et al., 2009), Cufflinks (Trapnell et al., 2010) and others (reviewed in Martin and Wang (2011))). Despite the success of such approaches, assembly artifacts remain a common phenomenon (Birney, 2011) and in the context of transcriptome assembly, many reads cannot be assigned to single isoforms unambiguously. When trying to interpret these reads, different sets of possible assemblies are theoretically compatible with the sequencing data (Garber et al., 2011) and this leads to the misassembly of false transcripts and a wrong estimation of specific isoform expression levels. This problem becomes more severe in the presence of complex alternative splicing patterns and depends on factors like the sequencing depth and the length of generated reads. These considerations are also the basis for recent developments that aim at identifying alternative splicing patterns from De-Bruijn graphs without actually assembling complete
isomers (Sacamoto et al., 2012) or methods that integrate RNA-seq data with existing gene annotations and ESTs to predict more reliable splice graphs and annotate ambiguous events as unresolved (Rogers et al., 2012). A systematic assessment of transcriptome assemblies is difficult since appropriate quality metrics have not been established yet and require a well-defined gold-standard that is difficult to find (Martín and Wang, 2011). During the revision of this paper, Anders et al. (2012) published a tool called “DEXSeq” for the detection of differential exon usage from RNA-seq data. Rather than using an assembly approach and comparing abundance levels of predicted transcripts, DEXSeq avoids the assembly step and calculates p-values for every annotated exon (or parts of exons if an exon modification event can be derived from the provided annotation). Similarly, our method directly identifies genes with significantly altered ratios of (potentially unknown) isomers. The power to detect significant expression differences depends on the number of available reads and therefore on the sequencing depth and length of the region of interest. Regions that are specific to a single isoform (like cassette exons) are often much shorter compared to full transcripts and the detection of significant differences may be severely hampered by their low number of reads. Besides this, the number of statistical tests that are required to detect previously known and unknown events for all expressed genes in the observed cells raises to such an extent that makes it challenging to avoid high false positive levels or to reach statistical significance after employing multiple testing correction. A critical aspect for all methods is the potentially very complex composition of isomers. Some genes generate many different transcripts in varying abundances that comprise a large variety of different combinations of exons. RNA-seq data gives the opportunity at hand to yield a global insight into this situation. Consequently, we developed a new method (and implemented it into the software tool “SplicingCompass”) to predict genes that show differential splicing taking into account all of their exons. To our knowledge, our method is the first approach that detects differential splicing on a gene-level by taking into account all exons of a gene at once instead of considering single exons independently. This reduces the number of statistical tests considerably and better accounts for combined effects. We applied our method to high-throughput sequences of the transcriptomes from human brain and liver samples as well as from neuroblastoma patients with low-risk and high-risk tumours and to simulated data. In addition, we present results that show the validity of our method as well as its applicability in the context of clustering tissues and cancer patients.

2 METHODS

2.1 Data

We analysed two RNA-seq data sets and compared high-throughput sequences of the transcriptomes from four human brain and seven liver samples (data published in Au et al. (2010); Blekhman et al. (2010) and via the “Human BodyMap project” from Illumina) as well as 14 neuroblastoma patients with low-risk and high-risk tumours (data published in Au et al. (2010) and via the “Human BodyMap project” from Illumina) as well as 14 neuroblastoma samples (data published in Au et al. (2010) and via the “Human BodyMap project” from Illumina) as well as 14 neuroblastoma samples as well as from neuroblastoma patients with low-risk and high-risk tumours and to simulated data. In addition, we present results that show the validity of our method as well as its applicability in the context of clustering tissues and cancer patients.

2.2 Mapping and pre-processing

TopHat (Trapnell et al., 2009) was used for a splice junction sensitive mapping of sequence reads to the reference genome UCSC hg19. We used default parameters but did not allow for multihits. To normalise reads for different sequencing depths we divided read counts by a library size parameter as described previously (Anders and Huber, 2010). To remove genes that are barely expressed, we calculated RPKM values (“reads per kilobase of exon model per million mapped reads”) (Mortazavi et al., 2008) on an exon-level and included genes that had at least one exon in the union transcript that was above a threshold of five RPKM averaged over the samples. Union transcripts were defined as described in the following section. We also excluded genes with only one annotated exon. With this, 127634 (79819) of 182291 exons in 10987 (6944) of 18121 annotated genes satisfied the criterion in the neuroblasto (brain/liver) comparison.

2.3 Splicing angles

For every gene g we defined a union transcript ut_g = (e_1,...,e_n) by combining all exons e_i from all corresponding isoforms that were annotated in Consensus CDS (CCDS) (Pruit et al., 2009). In the following, we represented the expression of that gene as a vector u_g, called read count vector. Every component of that read count vector corresponded to the number of sequencing reads R_i, that uniquely aligned with a given exon e_i of the union transcript: u_g = (R_1,...,R_n). Hence, u_g combined the expression levels of all isoforms of g. Systematic differences in alternative splicing affect the relative proportions between exon-expression levels and thereby determine the orientation of read count vectors. Hence, we used the geometric angles between read count vectors as a means to measure differences in alternative splicing. Furthermore, we added additional dimensions to u_g for all observed exon-exon connections based on junction reads found by TopHat (Figure 1). This generally increased the sensitivity of our method and helped to identify even small differences that arise from exon modification events like alternative 5′/3′ exons (see Supplementary Note 2 for a more detailed description). If a gene has a constant composition of isomers, i.e. the ratios between isoforms do not change between conditions, the read count vectors of that gene will be roughly parallel between conditions yielding a low angle between them, even if the overall gene expression levels differ (see Supplementary Figure 3). Therefore, our method inherently distinguishes between differences in overall gene expression and differences in alternative splicing.

2.4 Detection of differentially spliced candidate genes

To detect differentially spliced genes between two conditions c_1 and c_2 with n samples, we calculated all \binom{n}{2} pairwise angles as given in equation (1)

\[ \Phi_{n_1, n_2} = \arccos \left( \frac{\hat{v}_{n_1} \cdot \hat{v}_{n_2}}{|\hat{v}_{n_1}| \cdot |\hat{v}_{n_2}|} \right) \times \frac{180}{\pi} \]  

and tested if splicing angles within conditions were significantly smaller than splicing angles between conditions. \( \Phi_{n_1, n_2} \) is the angle between samples \( n_1 \) and \( n_2 \) for gene g, \( \hat{v}_{n_1} \) is the read count vector for gene g and sample \( n_1 \). We performed two tests per gene. To reduce false negatives from very high dimensional vectors, we restricted the dimensions of the read count vectors in the first test to exons for which a skipping or modification event was generally supported by junction reads and then used their pairwise splicing angles to test if these events were condition specific. Since alternative splicing events could not always be recognized from reported junction reads, we performed a second test that included all exons and all exon-exon junctions from the union-transcript. We assessed statistical significance with

\[ Transcript variation affecting the first/last exon(s) do not give rise to simple junction read patterns that normally span nested exons and would be missed with the first test alone. In addition, tools that discover splice junction reads still suffer from false negatives (Rogers et al., 2012).\]
pursued additional methods is largely assessed by simulations. We used the simulation engine to verify a subset of genes but in general the performance of prediction 

A major issue in verifying differential splicing predictions is the lack of an appropriate testing correction (Benjamini and Hochberg, 1995). A one-sided t-test and used the Benjamini-Hochberg procedure for multiple testing correction (Benjamini and Hochberg, 1995).

2.5 Verification of candidate genes

A major issue in verifying differential splicing predictions is the lack of an established gold-standard. Quantitative PCR can be used to experimentally verify a subset of genes but in general the performance of prediction methods is largely assessed by simulations. We used the simulation engine BEERS to simulate a variety of artificial differential splicing events but also pursued additional in silico strategies to assess the quality of our biological predictions. This included:

- Evaluation of splicing factor motifs which are essential to specifically enhance or silence the splicing of alternative exons in a tissue-specific manner (sections 2.6 and 3.2).
- Assessment of the number of publications found in an unbiased, automatic PubMed search (sections 2.7 and 3.3).
- Clustering of tissues and patients based on splicing information (sections 2.8 and 3.4).
- Assessment of different kinds of evidence of splicing diversity for predicted genes such as reads that span exon-exon junctions and exonic read coverage plots that were normalised for differences in gene expression (sections 2.9 and 3.1).

2.6 Splicing factor motifs

To detect splicing factor motifs, we used the R packages “Biostrings” and “BSgenome” (http://www.bioconductor.org) and scanned for all binding motifs characterized in the database SpliceAid2 (Piva et al., 2012). We used the “matchPattern” function without mismatches or indels and scanned for motifs around exon-intron boundaries (100 bps up- and down-stream) from all exons that were analysed with our method. For every splicing factor we calculated a confusion matrix comprising the number of exons with and without nearby motifs in predicted versus remaining genes. As a control, an identical analysis was performed with the same number of exons from random genes. We assessed statistical significances with Chi-square tests and corrected for multiple testing with the Benjamini-Hochberg procedure. P-values for differential expression of genes that encode for splicing factors were calculated from the RNA-seq data with DESeq (Anders and Huber, 2010).

2.7 PubMed search

We performed an automatic PubMed search and counted for every predicted gene the number of publications with a co-occurrence of the corresponding gene symbol and the term “alternative splicing”. A control search was performed with an equal number of random genes that were sampled from the same filtered gene set (i.e. only genes were sampled for the control that were actually tested by SplicingCompass and retained after expression and single-exon filtering). The control set was drawn such that the candidate set and control set were disjunctive and no gene was sampled twice (i.e. the predicted candidate genes were removed from the control set and sampling was done without replacement).

2.8 Clustering

To demonstrate that functional information was captured by SplicingCompass within the different splicing profiles, and not merely by differential gene expression, we performed an alternative splicing based hierarchical clustering of neuroblastoma patients and brain vs. liver samples with the genes identified by SplicingCompass. Analogously to the detection of differential splicing events, we clustered using all pairwise angles between samples. In addition, we then removed all genes that were significant in a gene-expression ranking based on the commonly used RPKM measure (i.e. we removed all genes with a RPKM based p-value ≤ 0.1) and repeated the clustering with this reduced set. For clustering, Pearson distance and average linkage was used in all cases.

2.9 Read coverage plots

To visually compare exonic read count profiles in terms of alternative splicing we normalised the number of reads \( R_{e_i;n_j} \) that mapped to all exons \( e_i \) from gene \( g \) in sample \( n_j \). We normalised for exon lengths and the overall gene expression level of \( g \) in a given sample \( n_j \) by

\[
R_{e_i;n_j}^{\text{norm}} = \frac{R_{e_i;n_j}}{l_{e_i} \cdot g_{n_j}^{\text{exp}}} \tag{2}
\]

In which \( l_{e_i} \) was the length of exon \( e_i \) and \( g_{n_j}^{\text{exp}} \) was the overall gene expression level of gene \( g \) in sample \( n_j \). We estimated \( g_{n_j}^{\text{exp}} \) by the median number of length-normalised reads that mapped to the exons of the union transcript from gene \( g \) in the given sample. Equivalently, we normalised and depicted the number of junction reads that TopHat reported between all expressed regions (i.e. known and unknown exons) of gene \( g \).

2.10 Comparison to DEXSeq and Cufflinks

We compared the predictions made by SplicingCompass, DEXSeq (Anders et al., 2012) and Cufflinks/Cuffdiff (Trapnell et al., 2010) for the described simulated and biological data sets. To compare DEXSeq with SplicingCompass (which assigns p-values to whole genes), we considered a gene differentially spliced in DEXSeq if at least one of its exons was
differentially used. For Cufflinks/Cuffdiff, we followed the steps described in (Trapnell et al., 2012) and considered a gene differentially spliced if any of the transcripts assembled for that gene was differentially expressed according to Cuffdiff (irrespective of the fact if the assembled transcripts correctly matched the simulated ones). Strictly speaking, this definition does not only include transcript variation produced by the splicing machinery but also transcript variation due to multiple promoter and polyadenylation sites.

3 RESULTS

3.1 Differentially spliced genes

We predicted 1595 genes to be differentially spliced between brain and liver samples and 24 genes to be differentially spliced between neuroblastoma stages (p-value ≤ 0.1 after Benjamini-Hochberg correction, Supplementary Tables 2 and 3). Supplementary Figure 4 exemplifies one top scoring gene predicted by our method that shows a clear exon exchange event. The event is strongly supported by exon junction reads and independent annotation information from the UCSC alternative splicing track (Karolchik et al., 2008). Read counts in the figure are normalized as described in Methods (section 2.9) and plotted for all exons of the union transcript from that gene. Exon seven is specifically skipped in liver tissues whereas exon eight is specifically skipped in brain tissues (SLC7A2 p-value = 10^{-22}). The splicing events are directly supported by splice junctions. According to UCSC, exons seven and eight are known to undergo alternative splicing. Another example that revealed a liver specific exon modification event in the transcripts of the gene MAGI1 is depicted in Supplementary Figures 5a and 5b. MAGI1 showed several condition specific splicing events that affected different exons. One of these events specifically shortened exon seven in liver samples. We used Ingenuity (Ingenuity Systems, www.ingenuity.com) and tested for gene set enrichment in the categories “Diseases and Disorders”, “Molecular and Cellular Functions” and “Physiological System Development and Function”. We found enrichments for “Neurological Disease”, “Cellular Growth and Proliferation” and “Cell Death and Survival”, which point at processes important for brain tissues while “Protein Synthesis” and “Nucleic Acid Metabolism” are central functions of the liver which also plays an important role in “Small Molecule Synthesis” and “Nucleic Acid Metabolism” are central functions of

3.2 Splicing factor motifs

During splicing, a protein complex known as the spliceosome assembles on the pre-mRNA to remove introns and join exons. In addition, a complex interplay between trans-acting splicing factors and auxiliary cis-acting regulatory elements takes place. This is essential to ensure accurate splicing and to achieve tightly regulated, tissue specific alternative splicing. Cis-acting elements can function as silencers or enhancers and are found in the vicinity of splice sites in introns and exons. In general, alternative splicing is determined by the combined effect of multiple positively and negatively acting elements and the fate of cassette exons is decided by the presence and arrangement of surrounding motifs as well as the condition specific ratio and modification status of splicing factor proteins (splicing regulatory principles are reviewed in Matlin et al. (2005)). Therefore, we investigated if a alpha of the phosphatidylinositol 3-kinase. It influences tumor cell growth and motility and is a potential therapeutic target in glioblastoma multiforme (Weber et al., 2011). Multiple isoforms are annotated for PIK3R1 that differ in the first six to eight exons. Our analyses suggest an increased expression of short isoforms that exclude the first six exons in stage 4S patients compared to stage 4 patients (see Supplementary Figures 6a and 6b). The short isoforms lack the SH3 domain that mediates specific protein-protein interactions (Koyama et al., 1993) as well as the Rho-Gap domain that influences different regulators of the cytoskeleton (Peck et al., 2002). CRTAC1 codes for the cartilage acidic protein 1 and can be used as a biomarker to discriminate chondrocytes from osteoblasts in humans (Steck et al., 2001). A previously unknown isoform of CRTAC1 (CRTAC1-B) has been identified in brain tissue and RT-PCR confirmed that both isoforms (CRTAC1-A and CRTAC1-B) are being expressed in human (Steck et al., 2007). The two isoforms differ by alternate last exon usage (i.e. the most 3’ exon) which matches our in-depth analyses of the read coverage derived from the neuroblastoma data (not shown). Only CRTAC1-A contains a functional O-glycosylation site resulting in a glycosylated isoform. Recent studies in mice showed that the unglycosylated isoform CRTAC1-B (also called LOTUS) binds to Nogo receptor-1 (NgR1) and inhibits the binding of Nogo-A (Sato et al., 2011). Nogo-A binding destabilizes the actin cytoskeleton of the nerve cell and triggers a growth cone collapse. This induced growth cone collapse leads to a stabilization of the CNS wiring at the expense of plastic rearrangements and regeneration (Schwab, 2010) supporting the hypothesis for functional relevance in neuroblastoma cells which originated from the primitive neural crest. Very recent studies suggested that the carboxyl-terminal region (UA/EC domain) is a functional domain of CRTAC1 responsible for NgR1 binding (Kurihara et al., 2012). In contrast to the 3’ exon of CRTAC1, which contains the O-glycosylation site, the exons coding for the carboxyl-terminal region (UA/EC domain) do not seem to be affected by alternative splicing directly. CRTAC1-A products could not be detected in mouse tissues by RACE experiments (Steck et al., 2007) suggesting that this isoform is not expressed in mouse. To our knowledge, a possible influence of the O-glycosylation motif on the binding to NgR1 has therefore not been investigated yet but seems to be a promising future aspect, in particular in the context of alternative splicing of neuroblastoma tumour cells.
significant number of exons with specific types of splicing factor motifs can be observed in genes predicted by SplicingCompass (Supplementary Figure 7). For 9 out of 62 splicing factors we found a significant enrichment or depletion of motif-associated exons (i.e. exons with at least one binding motif of the specific factor in 100 bps up- or downstream of the exon-intron boundaries) in our predicted genes from brain vs. liver tissues. This strongly suggests a special pattern of splicing regulation. For 7 of the 9 factors we found an enrichment of motif-associated exons in our gene list. The factors hnRNP K and SRp55 showed a significant depletion. Notably, hnRNP K has been described for its potential to act as a splicing silencer presumably by antagonizing the binding or activity of nearby enhancers (Revil et al., 2009). A depletion of silencer motifs might thus serve as a similar regulatory mechanism as an enrichment of enhancer motifs. As expected, a control analysis with the same number of exons from random genes did not yield any significant results. Many factors with enriched motifs did not show a significant difference in corresponding gene expression values. These factors might impose regulation in a more interrelated manner and corresponding protein activities may be regulated post-translationally. For instance, Sam68 showed the most significant enrichment of motif-associated exons in our data but did not show differential gene expression. Sam68 has been described as “a prototype regulator of alternative splicing whose function depends on protein modification in response to extracellular cues.” (Matter et al., 2002). Matter et al. showed in detail that phosphorylation of Sam68 is crucial in regulating the alternative splicing of a specific exon in CD44. For other factors that are differentially expressed but did not show an enrichment of motif-associated exons in the complete set of predicted genes, a distinct pattern of binding motifs could still be observed in single genes. For instance, we found an accumulation of motifs of the tissue specific splicing factor Fox-1 in the vicinity of differentially spliced exons from SLC7A2 (Supplementary Figure 4).

3.3 PubMed search

For 636 out of the 1595 candidate genes from the brain vs. liver comparison, our automatic literature search yielded at least one publication with a co-occurrence of the corresponding gene symbol and the term “alternative splicing”. This is significantly more than revealed by a control search with the same number of random genes (482 out of 1595, Pearson’s Chi-squared p-value: 1.4e-08). For 72 candidate genes we found more than 10 publications by automatic searching compared to 34 genes in the random set. Supplementary Figure 8 shows the number of publications found for our candidate genes and the control set.

3.4 Clustering results

Splicing differences can serve as powerful biomarkers to discriminate tissues and have a great potential to improve existing stratification methods of cancer patients. In addition, the ability to discriminate patients based on genes that do not show any distinct gene expression variation is supporting evidence for post-transcriptional differences (caused by splicing) in the gene set under consideration. For this reason our clustering analyses were also a means to test our method.

A hierarchical clustering analysis based on predicted genes yielded two main clusters which clearly separated all neuroblastoma patients according to their tumour stage (Supplementary Figure 9) and all brain from liver tissues (Supplementary Figure 10). Even after removing all genes with significant differences in their overall expression levels (i.e. we removed all genes with an RPKM based p-value $\leq 0.1$) we could robustly cluster tissues based on differentially spliced genes (Supplementary Figures 11 and 12). As expected, an RPKM based clustering with the same reduced gene set did not yield meaningful clusters any more (Supplementary Figures 13 and 14). This indicates that splicing angles actually exploit post-transcriptional splicing information which is not apparent in gene-level RPKM values. Correspondingly, p-values computed by SplicingCompass for differential splicing only showed little correlation to p-values computed by DESeq for differential expression (Pearson correlation of 0.27 (0.02) for brain vs. liver (neuroblastoma stage 4S vs. stage 4) comparisons (see Supplementary Figure 15). This further demonstrates that SplicingCompass does not merely detect differential gene-expression but, at the same time, supports findings that indicate some cross-talk between splicing regulation and gene expression regulation (Kornbluh et al., 2004).

3.5 Comparison to DEXSeq and Cufflinks

We compared our method with the two well established and well known methods DEXSeq (Anders et al., 2012) and Cufflinks/Cuffdiff (Trapnell et al., 2010). SplicingCompass and DEXSeq predicted a similar amount of differentially spliced genes for the brain/liver study. In brain vs. liver tissues, 1595 genes were predicted by SplicingCompass and 1489 genes by DEXSeq. In neuroblastoma patients, 24 genes were predicted by SplicingCompass and 8 genes by DEXSeq. Four of these genes were predicted with both methods and in the brain vs. liver comparison 873 of the genes were predicted with both methods (see Supplementary Figure 16). Cuffdiff did not report a significant expression difference between the Neuroblastoma stages for any transcript that was assembled by Cufflinks. For the brain/liver study, we ran Cuffdiff for 150 hours on 15 processing cores before we stopped the analysis. The estimated running time for Cuffdiff on eight processing cores is around 6 hours according to Trapnell et al. (2012) and we did not observe such excessive running times with the other data sets. In addition, we performed a comparison using simulated data in which we selected 30 genes and randomly “knocked out” half of their transcript isoforms (experiment 1) and a second experiment, where we focused on events where multiple exons were affected in an interdependent manner (experiments 2-4, see Methods). From the 30 genes in simulated experiment 1, SplicingCompass, DEXSeq and Cufflinks/Cuffdiff detected 23, 28 and 17 events respectively (see Supplementary Table 5). From the ten interdependent events simulated in experiments 2-4, SplicingCompass and DEXSeq detected all events with an isoform switch of 50%, eight vs. six events with an isoform switch of 30% and one vs. three events with an isoform switch of 10% (see Supplementary Table 6). Transcripts that were assembled by Cufflinks in the regions of the simulated true positive events were not reported as significant by Cuffdiff. See Supplementary Note 1 (b) for a more detailed description. Hence, SplicingCompass and DEXSeq showed a similar performance to detect these simulated differentially spliced genes, whereas Cufflinks/Cuffdiff did not report any of the events simulated in experiments 2-4.
Some of the genes found by SplicingCompass in the brain vs. liver comparison that were not detected by DEXSeq involved differential splicing of unannotated exons. E.g. SplicingCompass detected a liver specific exon in PCYT2 that was not annotated in CCDS when we downloaded the reference but, in the meantime, has been included in the current database (see Supplementary Figures 17a and 17b). SplicingCompass detected this event because it considers all reported junction reads (even between not annotated regions) while DEXSeq relies more on the provided annotation and therefore missed the event (see Supplementary Figure 17c). Providing DEXSeq with the most recent annotation in this case or generally with an annotation that is constructed from the sequencing data itself probably would have also allowed it to detect the event. However, this case demonstrates that SplicingCompass can even identify differential splicing that involves unknown exons if provided with appropriate junction read information. In other cases we saw strong evidence of differential splicing in genes predicted by SplicingCompass where DEXSeq did not reach statistical significance (see Supplementary Figure 18 for an example of such an event in PAPSS2 that was not reported by DEXSeq). In turn, DEXSeq reported significant exons in genes that were not significant in SplicingCompass. E.g. for NR1D1 (one of the four genes that was not reported by SplicingCompass in the neuroblastoma comparison), DEXSeq reported a differential usage of exon one. A relative coverage difference of exon one was also visible in the plots produced by SplicingCompass but the gene was not considered to be significant. This might be due to the fact that transcript variation affecting the first and last exons are generally less supported by junction reads which are used by SplicingCompass if possible. However, a differential usage of the last exon in CRTAC1 that was more distinct between neuroblastoma stages was detected by both methods in good agreement. While DEXSeq reports p-values on an exon level, the plots produced by SplicingCompass were often equally informative and in many cases suggested the same exons to be affected by differential splicing. In some cases, the plots produced by SplicingCompass even allowed for an interpretation of the underlying events that was more consistent with existing knowledge. E.g. in the case of PIK3R1 DEXSeq marked the first exon as differentially used (see Supplementary Figure 6c) while the plots produced by SplicingCompass allowed to infer three isoforms that differed in the first six to eight exons, an interpretation that is consistent with annotated transcripts in CCDS (see Supplementary Figures 6a/6b).

4 DISCUSSION AND CONCLUSION

We presented a new approach to predict genes that undergo differential splicing. SplicingCompass is able to detect complex and previously unknown events and inherently distinguishes between gene expression and splicing differences. Our method allowed us to detect tissue-specific alternative splicing in brain vs. liver samples and tumour stage-specific alternative splicing in neuroblastoma patients. We showed that a significant number of exons in our predicted genes are associated with specific splicing factor motifs and that a statistically significant number of publications link those genes to reported alternative splicing. Moreover, we successfully exploited splicing information to cluster samples of different tissues and neuroblastoma tumours. Our clustering results showed that we captured post-transcriptional modifications of genes which are not apparent in the general gene expression. Risk stratification of neuroblastoma patients is a major challenge that led to the development of expression classifiers based on mRNA levels (Oberthuer et al., 2006) as well as miRNA levels (De Preter et al., 2011). Our results bear the perspective of improved stratification methods for the diagnosis of cancer patients based on differential splicing. In eukaryotes, intron retention is the rarest type of alternative splicing, whereas exon-skipping is the most prevalent (Kim et al., 2007). SplicingCompass currently ignores coverage differences of introns but we plan to incorporate dimensions for observed retained introns in the next version of SplicingCompass. Mutations that cause aberrant splicing are known to be responsible for many diseases and at least 15% of all disease-causing, single base-pair mutations affect splicing (reviewed in Kim et al. (2008b)). The particular involvement of alternative splicing in cancer development has been demonstrated by splice variants of oncogenes and tumour suppressor genes that were found specifically in tumours from diverse tissues (reviewed in Venables (2004) and Hu and Fu (2007)) making alternative splicing analysis a very promising approach in cancer research. The increasing amount of RNA-seq data from cancer patients now paves the way for a broad application of splicing analysis tools in this respect. We identified several candidate genes in neuroblastoma patients with yet unknown or already reported functional relevance in tumour biology but it is clear that the differentially spliced genes together with their predicted effects have to be verified experimentally. While SplicingCompass can detect differentially spliced genes it does not aim to assemble the isoforms itself or infer isoform expression levels like assembly algorithms such as Cufflinks (Trapnell et al., 2010), Scripture (Guttman et al., 2010) and others. Transcriptome assembly from RNA-seq reads is very challenging especially in the presence of complex alternative splicing patterns that often occur in plants and mammalians. While assembly algorithms benefit from longer reads, algorithms that estimate gene-level expression values often recommend shorter reads (e.g. Li et al. (2010) suggests that 20 - 25 bases is optimal). This makes it difficult to choose the most appropriate sequencing protocol when a variety of analyses should be applied to the data. SplicingCompass works with all common read lengths as well as single and paired end data. However, exon junction reads and distance information of read pairs are well supporting the determination of the exact splicing events and isoforms involved. In addition, strand specific protocols are used more and more to preserve the strand information of reads which is usually lost during cDNA conversion (Levin et al., 2010). If the strand information for every read is available, reads can be unambiguously assigned to exons of genes annotated on the same strand. Our software comes with a plotting functionality that allows for the visual assessment of predicted genes (see Supplementary Figure 4). SplicingCompass enables to detect genes affected by previously unknown splicing events. We applied our method to a reference assembly for which unknown events can involve known exons from existing annotations. With appropriate junction read information, SplicingCompass can also detect unknown events involving exons not represented in the annotation. Alternatively, SplicingCompass can easily be combined with algorithms that define exonic regions from the sequencing data itself and might thus reveal additional unknown events involving newly defined exons.
SplicingCompass: differential splicing detection using RNA-Seq data.

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