Splicing mutation analysis reveals previously unrecognized pathways in lymph node-invasive breast cancer

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Somatic mutations reported in large-scale breast cancer (BC) sequencing studies primarily consist of protein coding mutations. mRNA splicing mutation analyses have been limited in scope, despite their prevalence in Mendelian genetic disorders. We predicted splicing mutations in 442 BC tumour and matched normal exomes from The Cancer Genome Atlas Consortium (TCGA). These splicing defects were validated by abnormal expression changes in these tumours. Of the 5,206 putative mutations identified, exon skipping, leaky or cryptic splicing was confirmed for 988 variants. Pathway enrichment analysis of the mutated genes revealed mutations in 9 NCAM1-related pathways, which were significantly increased in samples with evidence of lymph node metastasis, but not in lymph node-negative tumours. We suggest that comprehensive reporting of DNA sequencing data should include non-trivial splicing analyses to avoid missing clinically-significant deleterious splicing mutations, which may reveal novel mutated pathways present in genetic disorders.

Large-scale DNA sequencing studies have attempted to elucidate the genomic landscapes of breast cancer tumours to identify mutated genes and genomic variation that contribute to tumour development and progression¹⁻⁵. Typically, somatic mutations within gene coding regions are identified and then filtered for rare or novel variants predicted to affect protein structure or function⁶⁻⁹. Frequently mutated genes are cataloged, with the goal of inferring defective genes that are more likely to contribute to tumour phenotypes. However, there does not appear to be a consistent set of somatic driver mutations in most breast cancer cases. For instance, in 100 cases, 73 different combinations of abnormal gene sequences were reported⁴.

Some established cancer genes are enriched for mutations (i.e. TP53, PIK3CA, PTEN, MAP3K1, AKT1, CDH1, GATA3, MLL3 and RB1), in addition to genes that were not previously associated with breast cancer (including CBFB, RUNX1, TBX3, NF1 and SF3B1)¹⁰⁻¹². At least 49 genes (including known breast cancer genes) have been found to be significantly mutated, 16 of these reproducibly across multiple studies, and the majority were mutated in <10% of tumours.

Inconsistencies in mutation composition among different tumours present significant challenges to understanding the underlying etiology of tumour phenotypes. As a result of epistasis, mutations in genes with linked biochemical functions would be expected to reveal dysfunctional pathways in tumours⁸. Focusing analyses to one molecular subtype of breast cancer can also be useful in delineating dysregulated pathways that define the basis of tumour phenotypes⁴. Significant insight into tumour biology has come from selecting tumours with specific clinical identifiers, for example, by limiting mutation catalogs in metastatic tumours⁸⁰,¹¹.

Somatic mutation analyses of tumour exomes have focused on alteration of amino acid sequences, or highly conserved dinucleotides adjacent to exons, which usually impact mRNA splicing. Since these variants most likely comprise only a fraction of the total mutational load, the pathways inferred to be dysregulated in these tumours may be incomplete. For example, in familial breast cancer, variants of unknown significance have been explained by both experimental validation and in silico predictions of defects in BRCA 1/2 mRNA splicing¹²,¹³. Typically, genomic studies have used tools that predict splicing mutations based on the highly conserved dinucleotide sequences at mRNA 5’ donor and 3’ acceptor sites¹⁴. There are other well established methods that can identify splicing mutations beyond those directly at natural sites¹⁵⁻¹⁷, but these approaches have not been applied to genome-scale cancer studies, until recently¹⁸. Published studies have revealed only a small fraction of reported somatic mutations in cancer to be splicing mutations, accounting for only 2% of those reported¹⁻³. The present...
mRNA splicing was predicted as a result of 213 substitutions at containing 21, 11, and 9 exonic variants, respectively. Alteration of and TP53 alter mRNA splicing. 948 variants were found to affect both protein Shannon Pipeline predicted significantly more splicing mutations than the variant caller, Strelka 6 (see Supplementary Material section I for variants (SNVs) and 10,000 insertion/deletions (indels) detected using (Supplementary Table S1). There were 149,959 single-nucleotide Derivation of mutations

## Results

### Derivation of mutations.

Somatic mutations in 472 breast cancer tumours from 445 breast cancer patients were called using matched tumour-normal DNA exome sequencing data from TCGA (Supplementary Table S1). There were 149,959 single-nucleotide variants (SNVs) and 10,000 insertion/deletions (indels) detected using the variant caller, Strelka 4 (see Supplementary Material section I for results from an alternative variant caller and reasons for our selection of Strelka). Protein coding mutations were annotated by ANNOVAR 8 and splicing mutations with the Shannon Human Splicing Pipeline 18 (Table 1, see Supplementary Tables S2–4 for a list of all mutations). The Shannon Pipeline predicted significantly more splicing mutations than reported by TCGA, because the information-theoretic method employed enables analyses of variants beyond exon boundaries that alter mRNA splicing. 948 variants were found to affect both protein coding and splicing in 747 genes, among 319 tumours. DYN2CHL1, TP53 and P4D1 were the most commonly mutated of this group, containing 21, 11, and 9 exonic variants, respectively. Alteration of mRNA splicing was predicted as a result of 213 substitutions at synonymous codons among 139 tumours. Reanalysis of coding changes confirmed high concordance with the validated TCGA SNVs, however indels were less reproducible (Supplementary Table S5). Overall, 82.1% (n = 21,041) of protein coding mutations, and 86.5% (n = 371) of splicing mutations reported by TCGA were confirmed. A small subset of protein coding TCGA substitutions that were missed occurred within genes commonly mutated in breast cancer (35 TP53, 13 MLL3, 22 GATA3, 25 MAP3K1, 11 CDH1 and 10 PIK3CA; see Supplementary Table S6), however all splicing-associated SNVs found by TCGA in cancer-related genes were detected.

### Significantly mutated genes.

Significantly mutated genes were identified with the Mutational Significance in Cancer (MuSiC) software suite22. There were 225 genes with false discovery rates (FDR) of <0.05, based on the Fisher’s combined P-value (FCPT), convolution (CT) and likelihood ratio (LRT) tests. These results were compared with the 49 genes previously identified as significantly mutated14 (Supplementary Table S7). Among the previous genes reported by TCGA, TP53, CDH1, MAP3K1, and MLL3 were significantly mutated in this study by all tests, and AFF2, SF3B1, and CBFB were significant for the CT and LRT tests only. We additionally identified ARID1A as significantly mutated, concordant with an independent, large-scale, breast cancer genomics study4.

### Validating predicted splicing mutations.

Changes in mRNA splicing from the predicted mutations were validated with Veridical41, which corroborates predicted, aberrant splice isoforms by assessing mutation-derived sequence reads in tumour RNA relative to their abundance in controls lacking the mutation. Controls comprised tumours lacking a particular mutation (usually, n = 414) plus additional normal samples (n = 106). Of all variants analyzed from the 415 tumours with RNA-Seq data (n = 4,952), 988 variants (~20%) in 819 genes caused one or more splicing aberrations at significantly higher levels than in controls (p ≤ 0.05; i.e. intron inclusion, exon skipping, or cryptic splicing). Predicted natural splice site mutations (822 of 3,863, or 21.3%), were validated by abnormal mRNA isoforms more often than cryptic splice site mutations (166 of 1,089 or 15.2% variants). A total of 309 mutations were found to cause exon skipping, of which 163 (53%) led to expected frameshift mutations.

Sufficient expression levels for each gene, based on RNA-Seq coverage, were required for validation of mutations. An expression heat map, clustered by BC subtype, is shown in Supplementary Fig. S1. Variants occurring within significantly expressed genes (defined as an average of ≥20 reads per base) were statistically validated for 862 (27%) of 3,156 variants (p ≤ 0.05). Of 263 variants reported by TCGA in genes with at least this level of expression, 156 (59%) were validated by exon skipping (26 variants), by intron inclusion (80 variants), and by the combination of both types of evidence (50 variants, p ≤ 0.05).

Predicted cryptic splicing mutations were confirmed based on the presence of unique junction-spanning reads corresponding to the ectopically spliced isoforms in GATA3, PALB2, CBFB, ABL1, C2CD2L, ENSA, NASP, NOP9, and TFE3 (Supplementary Fig. S2). Four of these genes have been linked to tumourigenesis: ABL1, an oncogene, GATA3 and PALB2, which are associated with familial breast cancer6,23, and CBFB has been recently implicated in breast cancer by TCGA et al. and others14. These cryptic splicing mutations lead to short exonic deletions that alter the reading frame, and likely affect the activity of the gene products (Fig. 1). The GATA3 cryptic isoform is the only detectable transcript in the majority of controls, although it is substantially more abundant in the tumour sample (Supplementary Fig. S3).

The most commonly mutated genes with splicing mutations were also found by MuSiC to be significantly mutated in these tumours (n = 13, FDR < 0.05), and at least one third of the mutations were validated with RNA-Seq data (Table 2). In TP53, which exhibited the highest density of splicing mutations (Fig. 2), 18 of 23 (78%) predicted variants were validated to cause aberrant splicing (p ≤ 0.05).
All of the validated mutations exhibited statistically significant intron inclusion, and, in three instances, also resulted in exon skipping.

**Copy number analysis of mutated genes.** The validated mutations are organized and segregated by tumour subtype on a Circos plot (Fig. 3). Copy number changes portray the genomic locations of deletions or amplifications that coincide with these variants. Validated splicing mutations exhibit a relatively uniform genomic distribution, except for significantly mutated genes, such as TP53 on chromosome 17 and HMCN1 on chromosome 1. We investigated variants in regions showing copy number losses, which may constitute the “second hit” in oncogenesis. Of the 49 genes found to be significantly mutated in breast cancer, five contained splicing mutations (BRCA1 (2 tumours), PTEN (2 tumours), MAP2K4 (4 tumours), MAP3K1 (4 tumours) and KMT2C (7 tumours; also known as MLL3)) and also recurred within commonly deleted intervals. Of all genes with validated mutations in deleted regions, 9 harbored more than 2 variants: 1 had three, 4 had four, and only KMT2C possessed more than 4 variants.

**Analysis of pathways enriched in mutant genes.** Mutated genes were clustered by pathway overrepresentation analysis for protein coding (Supplementary Table S8, n = 202) and splicing mutations (Supplementary Table S9, n = 452). There were 100 pathways were common to both mutation sets (Supplementary Table S10). Pathways associated with all types of mRNA splicing mutations include those that affect collagen structural genes and enzymes that modify or metabolize collagen (n = 14, Supplementary Table S11 #1–14), and several that involve the extracellular matrix (ECM, n = 4, Table S11 #15–18). Many of these pathways (n = 17, Table S11 #1–13,15–18) are also overrepresented by pathway analysis of protein coding mutations.

**Relationship of mutation spectra to clinical findings.** Segregating splicing mutations by patient lymph node status revealed significant differences in mutated pathways between the two groups. Biochemical pathways with overrepresented mutant genes in lymph node-negative (LN-) vs. lymph node-positive (LN+) tumours are indicated in Supplementary Tables S12 and S13, and compared in Supplementary Table S14. There are 94 pathways overrepresented in both LN+ and LN- (Table S14 #421–514),

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![Table 2 | Genes Most Commonly Mutated with Splicing Mutations](image)

| Gene Symbol* | # Splicing Mutations | # Validated | % Validated |
|--------------|----------------------|------------|------------|
| TP53         | 24                   | 18         | 75         |
| HMCN1        | 19                   | 9          | 47         |
| KMT2C (MLL3) | 19                   | 7          | 37         |
| FHAD1        | 12                   | 4          | 33         |
| RAB3GAP1     | 11                   | 4          | 36         |
| BCLAF1       | 11                   | 3          | 27         |
| ANKEF1       | 10                   | 6          | 60         |
| RRM1         | 8                    | 4          | 50         |
| RRD1A        | 10                   | 2          | 29         |
| SCAMP5       | 7                    | 2          | 29         |
| CDH1         | 6                    | 4          | 67         |
| ACTR3        | 6                    | 2          | 33         |

*FDR < 0.05 for all genes from MuSiC (Fisher’s combined P-value, convolution and likelihood ratio tests).

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![Figure 1 | Cancer genes with validated cryptic splicing](image)

mRNA of ABL1, CBFB, GATA3 and PALB2, which each have validated cryptic splicing mutations confirmed using tumour-matched RNA-Seq data. Full gene lengths are displayed with vertical black bars outlining exon boundaries. The location of the cryptic variant is denoted by the red V, and the variant consequence is highlighted by white (wild type), dark grey (exonic deletion), and red (frameshift mutation). Conserved domains and protein interactions are labeled by the yellow and blue horizontal bars. In ABL1, the catalytic and C-terminal F-actin binding domains are disrupted. In PALB2, the region that interacts with BRCA2 is truncated. In the GATA3 aberrant transcript, the second zinc finger domain and a conserved motif crucial for DNA binding and protein function are affected by the altered reading frame.
including 17 collagen (Table S14 #421–437), and 3 ECM (Table S14 #438–440) pathways. Ontologically-related pathways were grouped (Supplementary Table S15) and visualized as Word Clouds (Fig. 4). Pathway groups overrepresented ($p < 0.05$) in both tumour subsets included 17 pathways involving collagen-ECM protein phosphorylation pathways, metabolism, cell cycle, DNA repair, and cellular response to stress. However, 13 pathways involving collagen (Table S14 #1–13), and 9 pathways involving NCAM1 (Table S14 #17–25) were overrepresented uniquely in LN$^1$ tumors, but not in LN$^2$ tumors.

NCAM1, or the neural cell adhesion molecule, is a member of the immunoglobulin super family with a role in cell-cell and cell-matrix interactions during development and cellular differentiation. Mutations in NCAM1 signaling genes for neurite outgrowth (Supplementary Table S16 #1) were still overrepresented in tumours with lymph node invasiveness, even after genes common to both tumour subsets were masked from the analysis, i.e. primarily collagen and ECM genes (Supplementary Tables S16–17). These include defects in NCAM1 interactions with FYN and GRB2, a ternary complex that participates in the conversion of RAS$^\text{GDP}$ to RAS$^\text{GTP}$, which subsequently initiates the RAF/MAP kinase cascade.

We then reanalyzed these data after conservatively limiting the set of mutant genes to those containing the most deleterious mutations (Supplementary Table S18; stop-gain, stop-loss, frameshift/indel mutations, and validated splicing mutations). Four of the 8 sub-pathways of NCAM1 signaling for neurite outgrowth were overrepresented solely in LN$^1$ tumours. Autophosphorylation/dephosphorylation of NCAM1-bound Fyn, as well as NCAM1-interactions with collagens were overrepresented. The most commonly mutated genes within these pathways are $SPTA1$, $CACNA1D$, $COL6A5$, NCAM1, and $COL6A6$ (Supplementary Table S19). $CACNA1D$ is a voltage-dependent Ca$^{2+}$ channel (VDCC) that associates with NCAM1 in growth cones at the sites of NCAM1 clustering. In addition, 6 other channel genes that are expressed in breast tissue were found to be frequently mutated ($CACNA1C$, $CACNA1D$, $CACNA1G$, $CACNA1H$, $CACNB1$, $CACNB3$). Mutations interrupting these VDCC interactions may alter the NCAM-dependent Ca$^{2+}$ influx. Collagen VI is expressed as supramolecular aggregates of composite structures of different chains and is among the most abundant components of the ECM. Knockdown of NCAM significantly reduces expression of ECM components, including collagen, weakening the ECM.
Mutations in these ECM components may also diminish matrix integrity, possibly resulting in more porous structures.

Elevation of NCAM1-related gene pathway mutations in lymph node-positive tumours. NCAM1, collagen, and ECM pathway mutations were assessed in tumours, stratifying by lymph node status and tumour stage (Fig. 5). The percentage of tumours with NCAM1-related pathway splicing mutations was increased in N0 (110 localized tumours) and N1 (84 tumours with lymph node involvement), as well as Stage I (37) and II tumours (140). Advanced lymph node involvement and tumour stage were not associated with increased numbers of collagen and ECM pathway splicing mutations, but rather a decrease in the percent of tumours with these pathways mutations in advanced stages was observed. A multiple factor analysis (MFA; Table 3) was performed to assess contributions of the number of NCAM1-related pathway mutations per tumour (both protein coding and splicing), clinical parameters including stage (AJCC tumour stage, lymph node status and metastasis stage), receptor status (HER2, PR, and ER positivity), and patient outcome (relapsed, living/deceased). NCAM1-related pathway mutations were either absent ($n = 213$), harbored a single mutation ($n = 117$), or two or more mutations ($n = 112$) per tumour. The MFA components containing NCAM1-related pathway mutations were moderately correlated with both tumour stage and receptor status, and accounted for 11% of the variance.
Analysis of tumour subtypes. Splicing mutation analysis in different tumour subtypes revealed between 9–15 mutations per tumour, which generally accounted for 8–9% of all mutations detected (Supplementary Table S20) and are similar levels to those previously reported 18. Pathway analyses for each subtype, stratified by lymph node status, indicated higher enrichment of NCAM1-related gene mutations in basal-like and HER2/ERBB2-enriched LN+tumours (Supplementary Table S21, Supplementary Fig. S5: see word clouds). LN+basal-like and HER2-enriched tumours were the only tumours found to have significant enrichment in "NCAM signaling for neurite out-growth", identifying those tumour subtypes and pathways that may play a role in tumour migration. No single gene was significantly mutated within the NCAM1 pathways that were overrepresented in LN1tumours. This suggests that a general defect in NCAM1-pathway signaling may be associated with lymph node metastasis in breast cancer.

Discussion
Breast carcinoma tumour exomes contain more deleterious mutations than previously recognized. Using Shannon information theory, we have predicted an expanded set of mutations that affect post-transcriptional mRNA processing that either reside in non-coding regions, or overlap known codons. We then employed Veridical24, a high-throughput, genome-scale method, to statistically validate mRNA splicing consequences that result from the predicted variants. This study complements the analyses performed by TCGA2, which comprehensively reported protein-coding mutations, along with gene expression, epigenetic, and copy number changes. Together with known deleterious coding sequence variants, the identification of such splicing mutations can refine and impact our understanding as to which biochemical pathways are dysregulated in these tumours.

Pathway overrepresentation analyses reproduced many of the same pathways identified by TCGA. In our analysis, a number of these attained or increased significance when genes with previously unrecognized splicing mutations were included. Both splicing mutations alone and the complete variant set from all tumours were enriched for genes in pathways known to play a role in tumour development and progression including signaling by growth factors, cell cycle, ECM organization, and cell-to-cell communication. Stratifying the tumours by lymph node status revealed that splicing mutations were enriched for genes within NCAM1 pathways in LN+ tumours, exclusively. Splicing mutations in these pathways were much rarer and sparsely distributed in LN−tumours, with 11 mutations in 92 LN2tumours and 25 mutations in 118 LN1tumours. Interestingly, this enrichment was not observed when all protein coding substitutions were analyzed, but was significant when assessing all variants that were likely to be deleterious (i.e. validated splicing mutations, stop codon gain or losses and frameshift substitutions). We did not attempt to differentiate loss versus gain of function, however splicing mutations and nonsense codons usually result in loss of function. The percent of tumours with NCAM1-related pathway mutations increased by 6% from lymph node stage N0 to N1 and N3 and by 7% from stage I to III. The lower fraction of tumours with collagen pathway mutations at higher lymph node stages (N3, N4), and with ECM-related mutations in tumour stages III and IV could be related to clonal selection of distinct metastatic phenotypes35, however it is also possible that the decreases may not be significant due to the lower numbers of tumours in these categories.

Our results indicate that NCAM1 pathways are more likely to be dysregulated in tumours that have migrated to lymph nodes. We found the enrichment of NCAM1-related pathway splicing mutations in LN+ tumours was specifically present in HER2-enriched and basal-like tumours. Basal-like, specifically triple-negative, tumours have been associated with poor prognosis and survival36. Early and metastatic HER2 positive tumours were associated with poor prognoses37 until the more recent introduction of HER2-targeted therapies38. In these tumour subtypes, the presence of NCAM1-related pathway mutations may indicate a propensity to migrate and/or form distant metastases.

Dysregulated expression of NCAM1 has been suggested to contribute to tumour migration in other cancers: (i) gene silencing and
localization studies have suggested that “NCAM is both necessary and sufficient to promote a migratory and invasive phenotype in EOC cells, with no major effect on cell proliferation” and, (ii) over-expression of NCAM1 has been linked to high ovarian carcinoma tumour grade and greater metastatic potential in melanoma cells; (iii) preserved NCAM1 expression in differentiated thyroid carcinoma has been cited as an indicator for tumours with as increased risk of forming distant metastases and (iv) blocking NCAM1 function in murine lung tumour cells led to cell vulnerability to apoptosis. More generally, NCAM1 is known to play a role in apoptotic evasion and matrix degradation, and has potential roles in directional cell migration, cell polarity, extravasation and immunological escape. NCAM1-mediated stimulation of FGFR activity is causally linked to tumour malignancy, suggesting that this NCAM1-FGFR interaction may be an effective therapeutic target. It is notable that we find mutations in breast tumours that affect the NCAM1-FGFR interaction occur in pathways that are overrepresented in LN+ , but not LN- tumour genomes.

NCAM1 homophilic clusters form within lipid rafts on the cell membrane. Spectrin, an NCAM1-binding cytoskeletal protein, colocalizes with NCAM1 and is codistributed within lipid rafts. Frequent mutations in spectrin (SPTA1) may prevent its association with the cytoplasmic NCAM1 domain, redistribution of NCAM1 and cluster formation. This could abrogate downstream interactions with FYN and GRB2, ultimately affecting activation of RAS. These findings merit further investigation into how dysregulation in these different partners (i.e. NCAM1, FGFR and the other interacting proteins), acting as an ensemble, may promote tumour metastasis.

The number of aberrant mRNA splicing mutations reported by TCGA is <10% of those reported here, and the variants were not functionally validated in the previous study. We predict that 8% of all cis-activating point mutations detected in these tumours will significantly reduce the strength of the corresponding natural splice sites. The 5,206 splicing mutations reported here nearly double the number of mutations that lead to stop-gains or losses (2,587 variants in 1,907 genes), and the number of insertions/deletions leading to frameshift substitutions (2,707 variants in 1,848 genes) in this set of tumours. It is not surprising that these analyses revealed previously unrecognized pathways that may be dysregulated, in addition to those already known in these tumours.

Our analysis of significantly mutated genes based on the protein coding and splicing mutations reproduced many of the genes reported by TCGA, and revealed one additional gene, ARID1A. ARID1A has been implicated in breast cancer in a large-scale genomic study and has also been mutated in 57% of ovarian clear-cell carcinoma tumours. Thirteen genes identified as significantly mutated in breast cancer by the TCGA did not reach statistical significance.
significance within our study (Table S4). This can be explained by a number of different factors: differences in variant callers, variant annotation, the number of tumours analyzed and differences in the filtering of variants, once the gene set was derived. In addition, TCGA initially analyzed all variants (SNVs and indels) by tumour subtype, unlike our study, which considered mutations in all tumours, then reanalyzed overrepresented pathways with mutations by subtype. Mutations that lead to a significant level of aberrant splicing can alter or improve genomic signatures, which are important when assessing potential biomarkers, diagnosis and prognosis, and metastatic or treatment-resistant tumour phenotypes.

**Methods**

This study involved an analysis of controlled-access data from The Cancer Genome Atlas Project (NCBI dbGaP Project #888) Predicting somatic genetic variants that alter the splicing of human gene transcripts, PI: PK Rogan). DNA and RNA breast cancer sequencing data was obtained for 445 tumours from 442 patients (Supplementary Table S1). DNA-Seq data was initially sequenced (July, 2012 DNA-Seq download; July, 2013 RNA-Seq Atlas Project (NCBI dbGaP Project #988: Predicting common genetic variants that affect RNA splicing and alternative splicing can alter or improve genomic signatures, which are important when assessing potential biomarkers, diagnosis and prognosis, and metastatic or treatment-resistant tumour phenotypes).

The tumour-normal pairs used mirrored those published by the TCGA in the Level 2 mutation data. Duplicate mutations in the same patient from two different tumour-normal pairs are reported, but were treated as one tumour for the mutation summaries reported by tumour. Somatic mutations were predicted from the same DNA sequencing data using two different algorithms: Strelka (v1.0.10) and SomaticSniper (v1.0.2) (See Supplementary Material section). Realignment was not performed in Strelka because of its alignment capabilities, so Strelka was run on the raw BAM files downloaded from TCGA.

Default parameters were used with the provided Burrows-Wheeler Aligner (BWA) configuration file, since BWA was used in the initial realignments. Additionally, the isSkipDepthFilters configuration option was changed to true, since such depth filters are redundant in a whole-genome dataset and would erroneously filter out most data when used with exome sequencing data. Strelka’s BWA quality control script was used to remove variants considered low quality. Variants that were found to be common SNPs, defined by those that were annotated with dbSNP135 in over 1% of the population, were filtered out from the variant set before any subsequent analyses.

Somatic mutations, including single-nucleotide variants (SNVs) and indels (indels) were used to predict the coding and non-coding genic effects of the variants. Annovar (August 23, 2013 release) was used with default parameters to predict which variants are likely to affect amino acid sequence and splicing at the natural splice sites. The Shannon Human Splicing Pipeline Version 2.0 (Shannon Pipeline) was used to complete a more in-depth analysis of splicing mutations, which predicts variants that will alter the binding affinity of the natural site or cause cryptic splicing (i.e. extension or truncation of an exon). The Shannon Pipeline results were subsequently filtered to prioritize which variants are most likely to have the greatest effect on mRNA splicing, using the filtering criteria outlined in Supplementary Fig. S6.

Multiple factor analyses used the R package FactoMiner (version 1.25) (30). Clinical parameters were obtained from the TCGA including AJCC tumour staging (metastasis stage code, neoplasm disease lymph node stage, and neoplasm disease stage), receptor statuses (estrogen, progesterone, and HER2/neu immunohistochemistry receptor status) as well as patient status (neoplasm cancer status and vital status). These clinical parameters were input into FactoMiner R as qualitative groups, as listed above, along with the number of NCAMI pathway mutations. Within the program, options were set to perform clustering after MFA, and to automatically determine the choice of the number of clusters. A second MFA was performed based on the number of NCAMI pathway mutations per tumour in genes present only in the NCAMI related pathways that were also not present in the collagen or extracellular matrix pathways.

Word Clouds were generated to portray the overrepresentation analysis of mutated pathways as depicted by Reactome(24) (Supplementary Fig. S24). The primary input data for these graphics was the overrepresented pathways from Reactome, partitioned by breast cancer subtype for this data using the R packages Hmisc (v3.14.3) and gplots (v2.12.1).

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Author contributions

P.K.R. and S.N.D. conceived of the study. P.K.R. directed the project, and S.N.D. and C.V. performed all analyses. S.N.D. and P.K.R. wrote and revised the paper, with input from C.V. All authors have approved the final manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: PKR is the inventor of US Patent 5,867,402 and other patents pending, which underlie the prediction and validation of mutations. He is one of the founders of Cytognomix, Inc., which is developing software based on this technology for complete genome or exome splicing mutation analysis. This software is available from Cytognomix (www.cytognomix.com).

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