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Mutational landscape of non-muscle-invasive bladder cancer

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Abstract

Non-muscle-invasive bladder cancer (NMIBC) includes stage Ta and stage T1 tumors and carcinoma *in situ* (CIS). Grading of Ta tumors sub-divides these lesions into papillary urothelial neoplasms of low malignant potential (PUNLMP) and low- and high-grade non-invasive papillary urothelial carcinoma [1]. CIS is by definition high-grade and the majority of stage T1 tumors are of high-grade. This pathologic heterogeneity is associated with divergent clinical outcome, with significantly worse prognosis for patients with T1 tumors or CIS. A wealth of molecular information has accumulated on NMIBC including mutational data that ranges from the whole chromosome level to next generation sequence data at nucleotide level. This has not only identified key genes that are mutated in NMIBC, but also provides insight into the processes that shape their mutational landscape. Although molecular analyses cannot yet provide definitive personal prognostic information, many differences between these entities promise improved disease management in the future. Most information is available for Ta and T1 samples and this is the focus of this review.

Gross chromosomal alterations

Since chromosomal banding techniques were developed, it has been clear that NMIBC often have near-diploid karyotypes in contrast to the high level of aneuploidy found in muscle-invasive bladder cancer (MIBC) [2]. Comparative genomic hybridization (CGH) and/or loss of heterozygosity (LOH) analyses later confirmed these early findings. Major differences between Ta and T1 tumors were revealed [3], with loss of 9q and Y identified as common events in Ta samples and a range of alterations including losses of 2q, 8p, 11p, gains of 6p, 8q and 11q and high-level amplification on 6p and 11q in T1. The latter alterations are also found in many MIBC [4]. Array-based CGH, SNP array and exome sequencing analyses now provide detailed genome-wide measures of copy number alteration at high resolution, confirming and extending these findings (Reviewed in [5]). Copy number clusters derived from an array-based CGH study including the entire grade/stage spectrum of bladder cancer [6] is shown in Figure 1, illustrating these marked differences in genomic stability.

Even in the absence of knowledge of the genes that are targeted by such copy number alterations, single chromosome alterations and overall copy number signatures provide prognostic information. The overall fraction of genome altered is associated with tumor grade, stage and prognosis [7, 8] and hierarchical clustering of copy number data can sub-classify both Ta and T1 samples into subtypes with different outcome [6, 8]. Two major copy number subtypes (GS1 and GS2) of Ta tumors have been defined [6]. GS1 subtype tumors show few copy number alterations, whilst GS2 are characterised by a very high frequency of 9q deletion accompanied by a range of other copy number changes, though these are not as complex as described in T1 or MIBC. Interestingly, although LOH of 9q is commonly found in regions of normal urothelium adjacent to tumors and in urothelial hyperplasia and is therefore considered to be a very early genomic alteration in bladder cancer pathogenesis, loss of 9q was not found as a single event but rather the most common event amongst a series of copy number changes. As no other common events were identified, this may suggest that loss of 9 rapidly leads to an increase in genomic instability. Stage T1 tumors also segregate

into at least two subtypes based on copy number profiles. In the single study reported to date, progression-free survival (PFS) was associated with T1 copy number subtype [8].

Candidate gene analyses

Candidates in regions of copy number alteration

The implication of copy number alterations is that tumor suppressor genes reside in regions of loss and oncogenes in regions of gain. Identification of the genes targeted by these alterations led to many studies during the 1980s and 1990s aimed at refining the candidate chromosomal regions and assessing candidate genes as valid contributors to bladder cancer development. Several key genes implicated in NMIBC were identified by candidate gene analysis during this period.

Chromosome 9 deletions are found in >50% of bladder tumors of all grades and stages and much effort has been made to identify the tumor suppressor gene(s) that drive this. In NMIBC, loss of the long arm (9q) is most common and the majority of tumors with deletion/LOH of 9q show loss of the entire chromosome arm, rendering deletion mapping to identify a common minimal region of loss difficult. Examination of a range of genes led the identification of *TSC1* (9q34) as the most plausible candidate. Mutations in *TSC1* are found in approximately 10-15% of bladder cancers with no clear association with grade or stage. As virtually all tumors with mutation have LOH of 9q34 and express no hamartin protein [9, 10], *TSC1* represents a classical two-hit tumor suppressor gene. In GS1 and GS2 subtype Ta tumors, that predominantly differ in retention or loss of 9q, transcriptome analysis has identified features that indicate a metabolic difference related to loss of function of one or both alleles of *TSC1* and consequent increase in activation of mTORC1 signaling [6].

The frequency of *TSC1* mutation is insufficient to account for the high frequency of loss of the entire chromosome arm, which may indicate that other genes on 9q contribute to urothelial tumor development. In addition to upregulated mTORC1 signaling, enrichment for expression of cell cycle and DNA repair genes was also found in tumors with 9q loss, though as yet no other candidate genes have been implicated in this. Interestingly, despite recent whole exome sequencing of reasonable numbers of NMIBC (see below), no gene on 9q apart from *TSC1* has been found with recurrent mutations. Thus, it is possible that the high frequency of deletion of the entire chromosome arm rather than the *TSC1* region alone implies that several genes on 9q are haploinsufficient in the urothelium.

Loss of 9p is found at lower frequency than loss of 9q in NMIBC. 9p deletions are focused on the *CDKN2A* locus (encoding p16 and $p14^{ARF}$), commonly with hemizygous deletion of the entire or a large part of the arm and focal homozygous deletion of the locus. Both hemizygous and homozygous deletions are more common in stage T1 than stage Ta tumors [11]. Compatible with this, loss of p16 expression is associated with reduced progression-free survival (PFS) [12]. A relationship between *FGFR3* mutation and *CDKN2A* deletion has been uncovered, such that deletions are more frequent in the context of *FGFR3* mutation in NMIBC. The presence of both alterations was associated with reduced recurrence-free survival (RFS) and PFS compared with those with *FGFR3* mutation alone. There is also a strong relationship between these events in the small number of MIBC that have *FGFR3* mutation, which implicates loss of *CDKN2A* in progression of NMIBC to MIBC [11].

Loss of 8p is associated with worse outcome in NMIBC [13, 14]. *SFRP1*, a negative regulator of WNT signaling shows promoter methylation in some cases and has been suggested as a candidate tumor suppressor gene in this region [13]. Infrequent focal amplification events (e.g 11q including CCND1, 12q including MDM2 and 8q including MYC) appear most common in high-grade and/or recurrent Ta tumors [15, 16].

Genes identified by candidate gene analysis

Analyses of specific candidate genes have revealed key oncogenic roles for *FGFR3, PIK3CA* and the RAS genes in NMIBC, all of which are most commonly activated by point mutation. The FGF receptor *FGFR3* (4p16.3) shows point mutation in up to 80% of Ta tumors. Mutation is much less common in T1 samples (10-30%) and MIBC (10-20%) [17-20]. These frequencies are higher than found in any other tumor type. Mutations are located in several hotspots, with the most common generating novel cysteine residues that are predicted to drive ligand-independent dimerization (Figure 2A). FGFR3 can also be activated by the generation of fusion proteins that retain the kinase domain of the gene fused in frame at the 3' end with another gene, commonly *TACC3* [21]. Most of the fusions reported to date have been in MIBC, but two have been found in cell lines derived from NMIBC and one in a stage Ta tumor [21-24].

PIK3CA, which encodes the catalytic subunit of phosphatidylinositol-3-kinase is also activated by point mutation in NMIBC, again at higher frequency than in MIBC [9, 25]. Overall, approximately 40-50% of Ta tumors and 6-20% of T1 tumors have mutation. Many mutations are found in hotspot codons E542, E545 and H1047, and these are known to activate the protein. However, a significant number of mutations, some with confirmed activation, have been found outside these codons (Figure 2B). Mutations in *HRAS* or *KRAS* are found in 10-15% of NMIBC. These do not show the same grade/stage distribution as those in *FGFR3* and *PIK3CA* but are evenly distributed across all tumor grades and stages. Mutations in these genes show an interesting pattern of co-occurrence and mutual exclusivity. RAS gene mutation is mutually exclusive with *FGFR3* mutation [26] but *PIK3CA* mutation is most commonly found with *FGFR3* or RAS mutation and rarely as a sole event. These relationships are most clearly seen in Ta tumors (Figure 2C). The precise explanation for mutual exclusivity of RAS and *FGFR3* mutation remains unclear. As both RAS and FGFR3 can activate the RAS-MAPK and/or PI3K pathways, this may be the explanation.

Mutations in the promoter of the telomerase reverse transcriptase gene *TERT* are the most frequent events identified in bladder cancers of all grades and stages. More than 70% of Ta and T1 tumors have mutations, largely confined to two hotspot nucleotides at positions -124 bp and -146 bp upstream from the ATG translation initiation codon [27-29]. Mutations have also been found in 65% of CIS samples [29], suggesting that this is a universal and early event in the development of both NMIBC and MIBC. These mutations can be detected with ease in patient urine samples [27-29], promising application in urine-based disease detection and monitoring. The mutations create novel ETS/TCF binding motifs, functional analysis of which indicates an effect on transcriptional activity [30]. A common polymorphism (rs2853669) within a pre-existing ETS2 binding site in the *TERT* promoter has been shown to act as a modifier of the effect of mutations on recurrence in NMIBC, such that patients with mutation and lacking the variant allele show reduced RFS [31]. Further examination showed that *TERT* mutations were associated with *FGFR3* mutation, with the majority of *FGFR3* mutant samples having *TERT* mutation. The presence of both mutations was associated

with shorter telomere length. Patients with NMIBC who carried the common rs2853669 allele and whose tumors had *TERT* mutation, had a two-fold higher risk of disease recurrence [32]. The explanation for the presence of the *TERT* mutation in most *FGFR3*-mutant tumors is not clear, but one possibility is that this is required in the presence of FGFR3-driven proliferation and associated telomere shortening.

TP53 has been extensively studied in bladder cancer. Mutation frequency is very low in low-grade Ta tumors but higher in T1 tumors [33, 34]. In a large study of T1 grade 3 tumors, 65.5% contained *TP53* mutations [35]. Little evidence for inactivation of the tumor suppressor genes *PTEN* and *RB1* has been found in NMIBC and this is now confirmed by whole exome analysis (see below). A tumor suppressor with significant mutational inactivation identified by candidate gene analysis in NMIBC tumors is *STAG2.* Mutations have been detected in 30-36% of Ta and 18-27% of T1 samples [36, 37]. Although STAG2 is a component of the cohesin complex that regulates chromatid separation, loss of this function is not implicated in bladder cancer mutant samples which represent the least genomically unstable group [36, 38]. Therefore, the effects of loss of function are predicted to relate to other functions such as regulation of gene expression, DNA replication and repair [39].

Mutations identified by whole exome sequence analysis

Several recent exome sequencing and/or targeted re-sequencing studies include or have focused entirely on NMIBC [6, 24, 38, 40-42]. Findings reveal significant differences from MIBC and provide a comprehensive view of the mutation spectrum of NMIBC at nucleotide level.

Mutation spectrum

Whole exome sequence analysis has reported mean and median somatic mutation rates of 2.4 and 1.6 per megabase (Mb) respectively in Ta tumors [6]. Currently there is no data for T1 tumors alone but in a mixed sample series with predominantly Ta and T1 samples rates were not significantly higher than for Ta tumors alone [42]. This mutational burden is significantly lower than the mean and median non-synonymous somatic mutation rates of 8.2 and 5.8 per megabase (Mb) reported for MIBC [43]. The overall (i.e. synonymous and non-synonymous) number of exonic single nucleotide variants (SNVs) in NMIBC shows a broad range. In a series of primary Ta samples consisting almost entirely of grade 1 and 2 tumors, an average of 119 (range 37-283) was found [6]. Two mixed sample series report similar findings; 195 (range26-799) in a series containing 20 Ta, 5 T1 and 5 T2 [42] and 169 (range 4-360) in a series containing 6 Ta, 9 T1 and 2 T2 samples [38]. Of these SNVs, 20-50% were synonymous and the remainder potentially damaging.

Exome sequencing studies have found that in NMIBC as in MIBC, C>T transitions are the most common substitution (~50%), followed by C>G transversions (~25%) [6, 38, 41, 42]. Examination of the 5' and 3' context of the variants has revealed that as in MIBC [43], there is a strong bias towards a signature generated by the APOBEC family of cytosine deaminases. These enzymes show specificity for the motif TCW, where W is T or A [44]. A large proportion of NMIBC is enriched for this signature [6, 41, 42, 45, 46]. When the two Ta copy number subtypes defined by Hurst *et al* [6] were compared, the more genomically complex subtype, GS2, showed significantly higher mutation burden and APOBEC signature enrichment than GS1. This is in accord with the finding that the signature is higher in recurrences of NMIBC [41]. Expression of

APOBEC3A and APOBEC3B was associated with one of the three NMIBC expression subtypes (class 2) defined in the large UROMOL study that included both Ta and T1 tumors [46] and this correlated with presence of the APOBEC signature. In primary Ta tumors classified according to DNA copy number, APOBEC3H mRNA levels showed association with copy number subtype GS2 [6].

Mutated genes

Data for genes with mutation frequencies greater than ~10% in Ta tumors and for selected genes that are more frequently mutated in T1 tumors is presented in Table 1. The high frequency of *FGFR3, PIK3CA* and *STAG2* mutations is confirmed and recent studies reveal a major role for inactivation of chromatin modifier (CM) genes. These genes include the histone lysine demethylase *KDM6A,* the histone methyl transferases *KMT2A, KMT2C* and *KMT2D*, a SWI/SNF-related gene, *ARID1A* and histone acetyltransferases *EP300* and *CREBBP*. A panel of Ta tumors analysed by WES all contained mutations in $\geq$2 CM genes (range 2-16), many of which were clearly inactivating (indels, nonsense, splice site) and in the related prevalence screen using targeted sequencing, 91% of tumors had mutation in at least one of the 17 CM genes analysed [6].

KDM6A is more commonly mutated in NMIBC than MIBC. It encodes a histone 3 lysine 27 di- and tri-methyl (H3K27me2/me3) demethylase that in complex with KMT2D/KMT2C acts to create transcriptionally active chromatin conformations. This complex antagonises Polycomb-repressive complex 2 (PRC2) that contains the histone methyl transferase EZH2. Loss of KDM6A function is expected to lead to gene silencing. Compatible with this, comparison of expression profiles of WT and mutant bladder cancer samples has revealed more downregulated than upregulated pathways and enrichment of signatures associated with PRC2 repression in mutant samples [47]. An interesting finding is that although *KDM6A* is an X-linked gene that does not show inactivation, the mutation frequency in Ta samples from females appear significantly higher than in those from males [6]. If these findings are confirmed, this may suggest that the epigenetic landscape and vulnerabilities of the male and female urothelium differ and that this influences the molecular landscape of Ta tumors. Such a gender-related bias is not apparent in data from MIBC [43].

Other novel genes that show different mutation frequency in NMIBC and MIBC include *RHOB* and *RBM10.* Mutations in the small GTP-ase *RHOB* were reported in 13% of Ta samples [6] compared with 6% in MIBC [43]. Most were missense mutations clustered in "hotspot" regions of the protein that have been implicated in interaction with the protein-kinase-C-related kinases PKNs 1-3 [48]. Several of the missense mutant forms were shown to have reduced half-life, indicating a tumor suppressor role. In addition to regulation of the actin cytoskeleton and cellular migration, RHOB plays a role in inducing responses to a variety of cellular stresses and its loss can reduce DNA repair capacity and apoptosis in response to genotoxic stimuli (Reviewed in [49]). Loss of these functions may be highly relevant during development of NMIBC.

RBM10 (Xp11.3) encodes an RNA-binding protein and splicing regulator. Inactivating mutations appear to span the entire grade/stage spectrum of NMIBC (Table 1). Knockdown of RBM10 leads to altered splicing of a wide range of genes in cancer cell lines, several of which are associated with RAS signaling [50]. The effects of loss of function are likely to be cell type specific and at present the mRNA species affected in NMIBC are unknown.

Several DNA repair genes are implicated, including *ERCC2, ATM, ATR, BRCA1, BRCA2, POLE* and *FANCA* with mutation found more frequently in high grade Ta and T1 tumors [24, 38]. In a targeted sequence analysis, there was a clear relationship of mutations in the nucleotide excision repair gene *ERCC2* to higher numbers of mutations per Mb in the coding regions sequenced [24]. In MIBC, *ERCC2* mutation has been associated with a distinct spectrum of single nucleotide mutations [51] but this has not yet been assessed in NMIBC.

Several genes reported to be mutated in NMIBC have not been adequately analysed to determine how significant their role may be. These include *NF1, FAT1, ERBB2, ERBB3* and *ELF3,* all of which have functions compatible with a role in tumor development [24, 42]. As for the DNA repair genes, data for most of these indicates more frequent mutation in high grade and/or T1 samples. *ERBB2* missense mutations were found in 4% of Ta low-grade samples compared with 18% of Ta high-grade, CIS and T1 samples and these were mutually exclusive with *FGFR3* mutations. Six of 12 CIS samples analysed had mutations [24].

As has been shown by many molecular analyses over the past decades, genome sequencing studies indicate that at all levels, the mutational landscape of NMIBC is significantly different from that of MIBC. This is most striking for Ta tumors. A comparison of mutation frequencies of genes mutated at >10% in MIBC and primary Ta samples is shown in Figure 3.

Tumor evolution and intra-tumor heterogeneity

Sequencing of synchronous multifocal NMIBC, metachronous tumors from the same patient and samples before and following disease progression has been reported [41, 42, 45, 52]. Initial tumors that later progressed, have been reported to show higher levels of intra-tumor heterogeneity in mutational profile and more APOBEC-related mutations than those that did not progress, implying that APOBEC mutagenesis contributed later in tumor progression. Phylogenetic analysis showed that these tumors shared a monclonal origin [41]. In another analysis, paired samples pre- and post-progression showed increased numbers of mutations and divergence in SNVs, indels and breakpoint content in the progressed tumors, and few re-arrangements in common with the related pre-progression samples. However, the ancestral clones contained mutations in several genes that are commonly mutated in NMIBC (*FGFR3, KDM6A, PIK3CA*), confirming monoclonal origin. In all cases, a minor subclone from the primary tumor had expanded in the progressed tumor [42].

Future outlook

Treatment options for NMIBC are currently limited to transurethral resection and localised intravesical chemotherapy or BCG, and are guided primarily by the tumor stage and histopathology. The efficacy of these treatments is relatively poor and until recently little progress has been made in identifying new therapeutic approaches. Knowledge of the molecular landscape of bladder cancer has been greatly enhanced by the use of whole-genome technologies. These studies are revealing clinically actionable alterations (activating mutations, amplifications and fusions) and high frequencies of alterations in chromatin modifier genes which may guide novel and emerging approaches to intravesical therapy. They may also greatly

enhance the ability to predict disease course and treatment response when used in combination with existing clinical risk factors.

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Table 1. Mutations identified in stage Ta and stage T1 tumors by whole exome and targeted next generation sequencing.

	Hurst *et al* 2017	**Pietzak *et al* 2017**	**Pietzak et al 2017**	**Nordentoft *et al* 2014**	**Balbas-Martinez *et al* 2013**	**Pietzak et al 2017**	**Balbas-Martinez et al 2013**	**Guo *et al 2013***	**Meeks *et al* 2016**
	n = 82	n = 23	n = 32	n = 20	n = 33	n = 38	n = 32	n = 32	n = 25
	79 Ta G1/G2 + 3 TaG3	Low grade Ta	High grade Ta	Ta G1/G2	Ta G1/2	T1	T1	T1	22 T1 G3 + 3 Ta G3
Gene									
FGFR3	79	83	59	40	34	34	9	25	28
PIK3CA	54	39	34	25	16	21	6	6	36
KDM6A	52	61	47	65	16	45	9	53	24
STAG2	37	39	12	25	19	21	3	25	8
KMT2D	30	22	37	15	25	18	3	0	28
ARID1A	18	17	34	35	9	26	22	6	32
EP300	18	17	22	25	12	8	6	16	16
KMT2C	15	13	19	30	9	5	3	3	12
CREBBP	15	9	34	20	19	18	12	12	0
RHOB	13	ND	nd	0	(1)	nd	(0)	0	ND
HRAS	12	0	3	10	0	8	3	19	0
KMT2A	11	4	47	0	6	10	9	9	4
TSC1	11	0	16	5	(0)	10	(0)	12	8
BRCA2	10	9	9	0	0	13	3	0	0
COL11A1	10	nd	nd	0	(0)	nd	(0)	0	ND
RBM10	10	9	12	20	(1)	8	(0)	0	0
TP53	4	4	16	5	9	34	22	25	60
RB1	(0)	0	0	5	0	8	6	9	24

KRAS	2	17	6	0	ND	8	ND	6	12
ELF3	(4)	ND	ND	25	ND	ND	ND	12	ND
ERCC2	(4)	4	34	0	ND	13	ND	6	ND
NF1	(0)	4	16	0	ND	10	ND	6	0
TERT promoter	ND	61	88	ND	ND	79	ND	ND	68

Top panel; genes with >10% mutation frequency in the largest study of Ta samples. Lower panel; selected genes that are mutated at significant frequency in studies of T1 tumors.

Numbers indicate % of tumors containing one or more mutations.

Numbers in brackets indicate percentage found in exome sequence data series for genes not included in related targeted analysis.

Hurst *et al* 2017: 24 Ta samples were exome sequenced and 58 analysed by targeted profiling. Series included 3 grade 3 Ta. In this study FGFR3was assessed independently by SNaPshot analysis.

Balbas-Martinez *et al* 2013: 5 low grade Ta were exome sequenced and 20 analysed by targeted profiling; one high grade Ta was exome sequenced and 7 by targeted profiling. 9 T1 samples were exome sequenced 23 analysed by targeted mutation profiling.

Samples in Guo *et al* and Nordentoft *et al* were all exome sequenced.

Figure Legends

Figure 1. Copy number profiles of bladder tumors showing relative genomic stability of NMIBC

Unsupervised hierarchical cluster analysis of copy number data from 160 tumors of all grades and stages. Columns represent samples and rows genomic position. Blue, copy number gain; yellow, copy number loss; black, normal copy number. Chromosome number is shown on the left-hand side. The stage (white box, Ta; grey box, T1; black box, ≥T2) and grade (purple box, G1/2; orange box, G3) of each tumor is shown at the top of the figure.

Figure 2. *FGFR3, PIK3CA* and RAS gene mutations in NMIBC

A. Schematic of FGFR3 protein (IIIb isoform) and corresponding exonic positions. Codons showing activating point mutation and approximate frequencies as percentage of mutations reported in NMIBC are indicated. SP, signal peptide; IgI, IgII and IgIII, immunoglobulin-like domains; AB, acid box; TM, transmembrane region; TK, tyrosine kinase.

B. Schematic of PIK3CA protein showing approximate frequencies of mutations reported in the literature in hotspot codons (indicated in orange). Other mutation shown were identified by exome and targeted mutation analysis of stage Ta tumors [6].

C. Pie chart showing distribution and combinations of mutations in *FGFR3, PIK3CA* and RAS genes in stage Ta tumors. Data from [6].

Figure 3. Comparison of mutation profile of muscle-invasive (≥ stage T2) and stage Ta bladder tumors

Non-synonymous mutation frequencies in muscle-invasive bladder cancer (MIBC) [43] and in non-invasive bladder cancer (NIBC; stage Ta) [6], where one or other cohort had a reported frequency of >9%. Zero frequencies for NIBC indicate absence of mutation in the relatively small discovery series analysed and lack of inclusion in subsequent targeted mutation screening.

Figure
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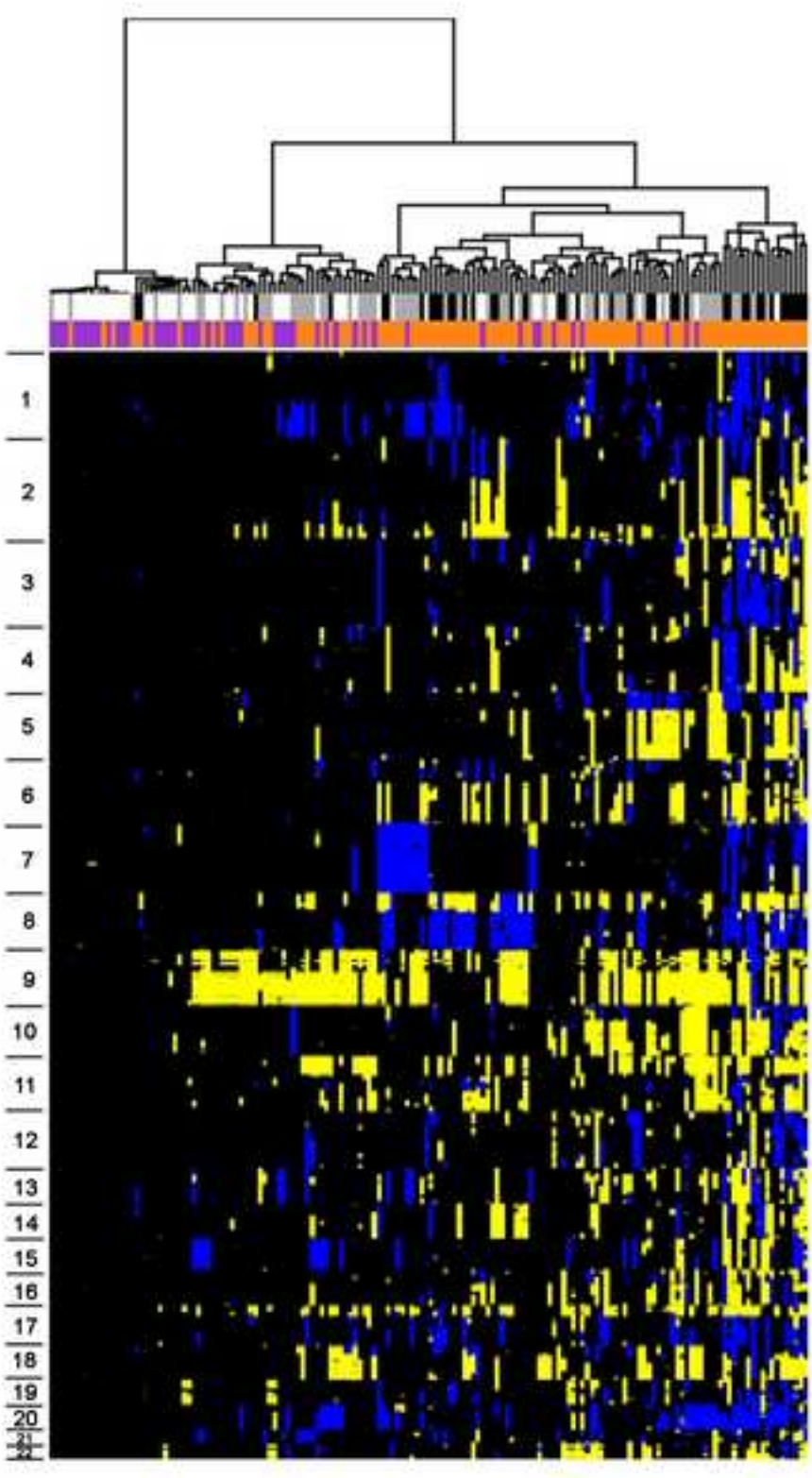

Figure 1

Figure

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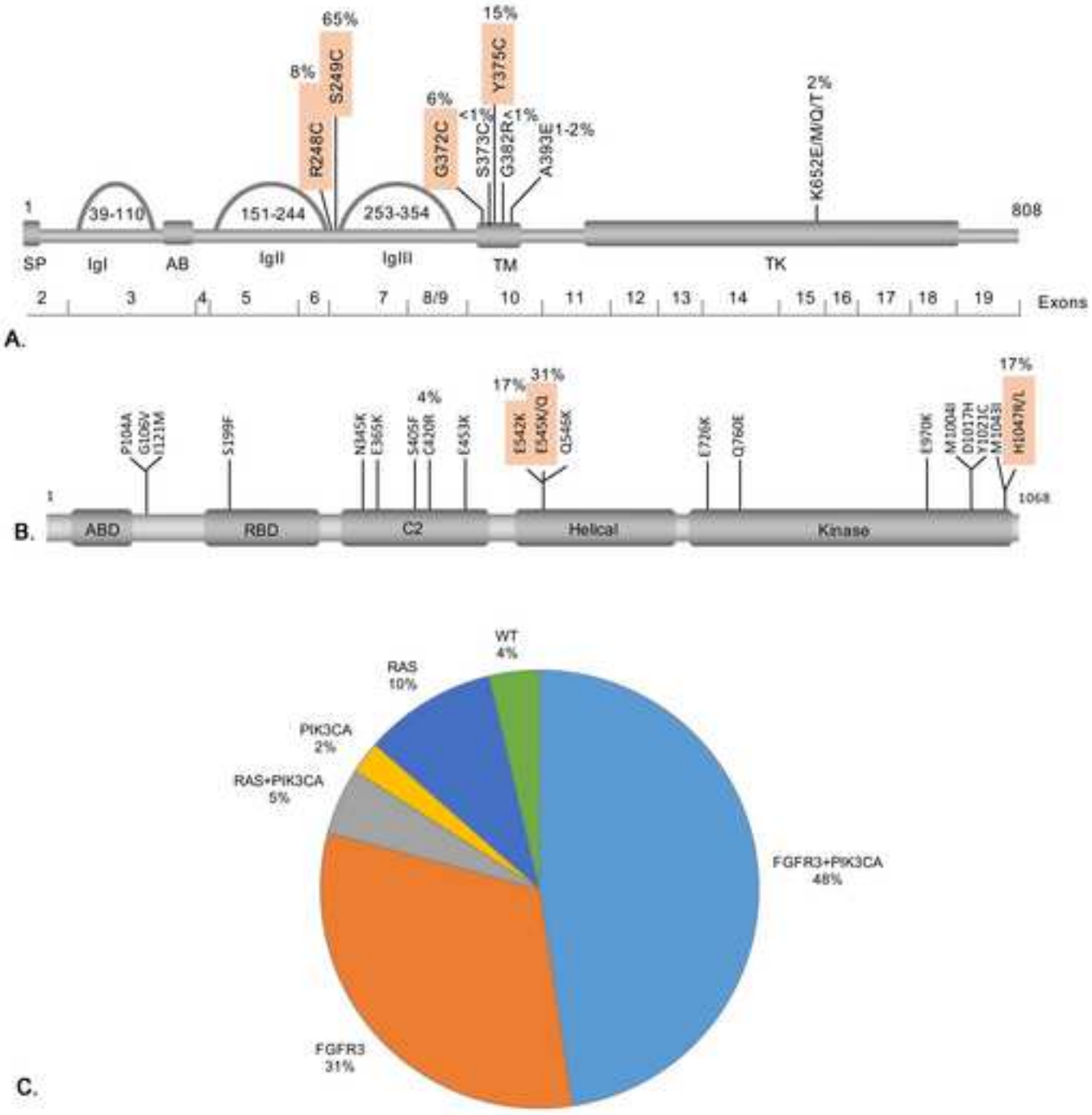

Figure 2.

Figure
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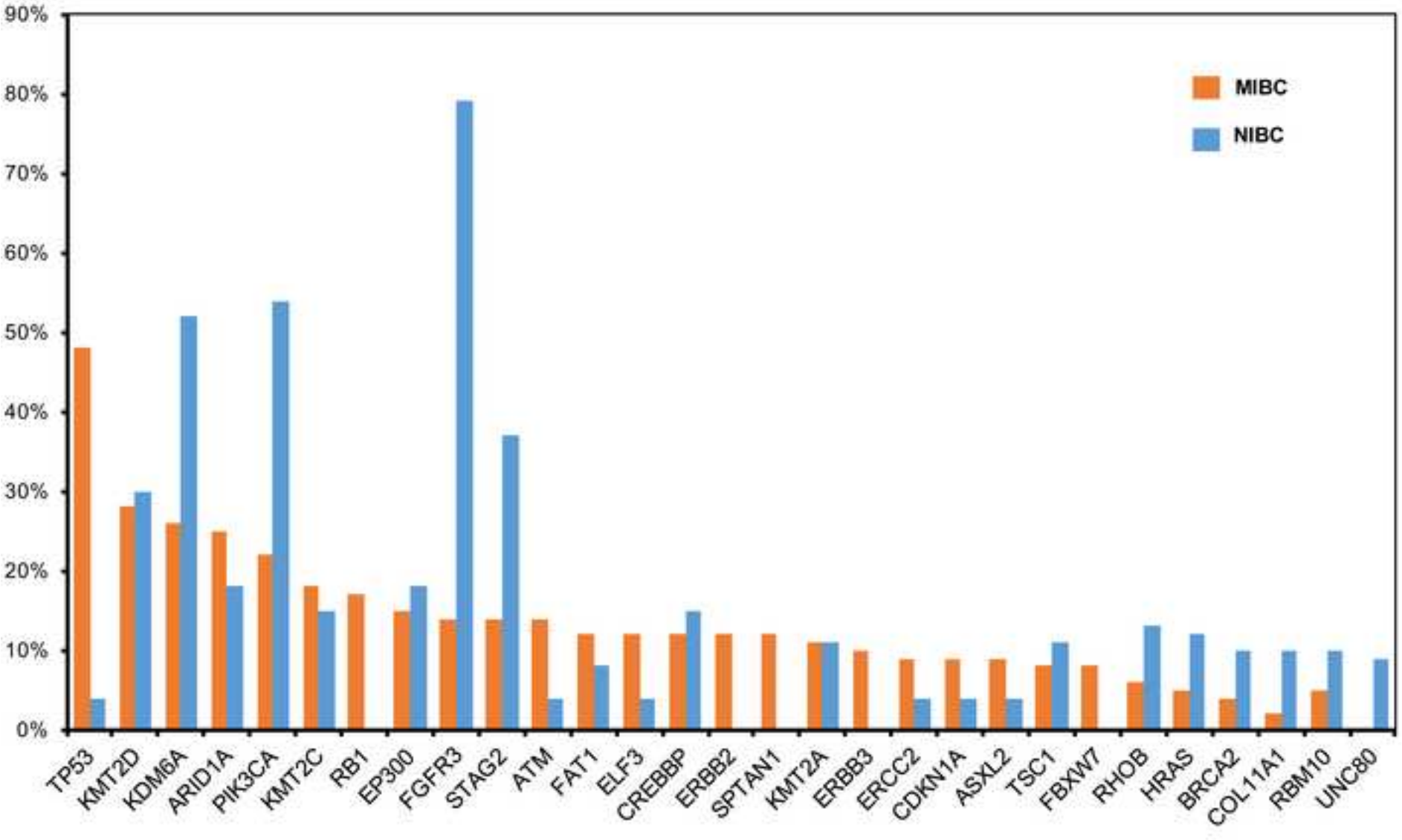

Figure 3