

# Tumorigenic cis-regulatory mutations in neuroblastoma

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**Background** | Neuroblastoma is a paediatric tumour of the peripheral sympathetic nervous system originating from the neural-crest cells. It is the second most common childhood solid cancer and its cure remains a challenge. In recent years, next generation sequencing of neuroblastoma has documented low somatic mutation rates and few recurrently mutated genes. As a result, the search for therapy targets is limited. Furthermore, as most studies on neuroblastoma relied mainly on whole exome sequencing, the role of somatic mutations in non-coding regulatory regions remains underestimated. Moreover, the growing interest in non-coding cis-regulatory variants as cancer drivers is currently hampered by numerous challenges and limitations of variant prioritization and interpretation methods and tools.

**Aims** | We hypothesized that mutated active regulatory elements could de-regulate genes involved in the tumorigenesis of neuroblastoma.

**Methods** | To overcome the limitations of noncoding driver analysis, we focused on active cis-regulatory elements (aCREs) to design a customized panel for deep sequencing of 56 neuroblastoma tumor and normal DNA sample pairs. We defined CREs by a reanalysis of H3K27ac ChIP-seq peaks of 25 neuroblastoma cell lines. Common H3K27ac peaks represented our target in which to search for driver mutations. We tested these regulatory genomic regions for an excess of somatic mutations and assessed the statistical significance with a global approach accounting for chromatin accessibility and replication timing. Additional validation was provided by analyzing whole-genome sequences of 151 neuroblastomas. HiC data analysis was used to determine the presence of candidate target genes interacting with mutated regions. We also used the k-means clustering algorithm to divide transcriptomic data of 498 neuroblastoma samples into two groups based on expression levels of genes that (according to the HiC results) significantly interacted with mutated aCREs. Moreover, we conducted a motif analysis to assess whether the somatic variants within the selected aCREs disrupted or created transcription factors binding motifs.

**Results** | We identified a significant excess of somatic mutations in aCREs of diverse genes including IPO7, HAND2, and ARID3A, and used the luciferase reporter gene assays and the CRISPR-Cas9 editing to assess the functional consequences of the mutated IPO7 aCRE on candidate target genes (IPO7, TMEM41B, DENND5A) ( $P < 1.0 \times 10^{-03}$ ). Taken together, patients with noncoding mutations in aCREs showed inferior overall, and event-free survival ( $P < 2.0 \times 10^{-03}$ ). By multivariable analysis, we confirmed that the noncoding mutational burden was independent of age at diagnosis, tumor stage, risk group, and MYCN status ( $P < 2.0 \times 10^{-02}$ ). We also found that the expression profiles of many of the aCREs target genes (tested singularly and in a combined manner) associated with markers of unfavorable prognosis and low survival rates ( $P < 5.0 \times 10^{-02}$ ). Furthermore, we conducted a motif analysis to identify transcription factors with altered binding motifs. Overall, the biological functions of aCRE target genes and those of transcription factors with mutated binding motifs converged towards processes related to embryonic development and immune system response ( $P < 5.0 \times 10^{-02}$ ). This suggests that the combined effect of noncoding cancer driver mutations is the alteration of gene sets involved in specific molecular mechanisms underlying neuroblastoma tumorigenesis.

**Conclusion** | We integrated multiple data levels taking epigenomics, genomics and transcriptomics information of neuroblastoma to set up an alternative approach for detecting and studying regulatory cancer driver mutations. Our strategy enabled us to identify mutated regulatory regions that may play an important role in regulating biological processes associated with tumor development and immune escape.

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