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Inversion-mediated gene fusions involving *NAB2-STAT6* in an unusual malignant meningioma

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Background: Meningiomas are the most common primary intracranial tumours, with ~3% meeting current histopathologic criteria for malignancy.

Methods: In this study, we explored the transcriptome of meningiomas using RNA-Seq.

Results: Inversion-mediated fusions between two adjacent genes, *NAB2* and *STAT6*, were detected in one malignant tumour, creating two novel in-frame transcripts that were validated by RT-PCR and Sanger sequencing.

Conclusion: Gene fusions of *NAB2-STAT6* were recently implicated in the pathogenesis of solitary fibrous tumours; our study suggested that similar fusions may also have a role in a malignant meningioma with unusual histopathologic features.

Meningiomas are primary central nervous system tumours that originate from the meningeal coverings of the brain and spinal cord. They represent up to 30% of all primary intracranial tumours (Riemenschneider *et al*, 2006). Grades I, II and III meningiomas as classified by the World Health Organisation (WHO) are typically referred to as benign, atypical and anaplastic or malignant, respectively (Perry *et al*, 2004). Malignant meningiomas are typically associated with early recurrence, possible metastasis and decreased overall survival (Perry *et al*, 1999; Mawrin and Perry, 2010). Although multimodality therapy including maximal surgical debulking, radiation and chemotherapy are frequently used to treat malignant meningiomas, outcomes for this disease remain poor.

Genomic instability is associated with progression of meningiomas. Copy number variation was found to correlate with defined grades of tumours (Lee *et al*, 2010). Mutations of *NF2*, *SMO*, *AKT1*, *SMARCE1*, *MEG3*, *TRAF7* and *KLF4* genes have been identified in meningiomas (Christiaans *et al*, 2011; Balik *et al*, 2013; Brastianos *et al*, 2013; Clark *et al*, 2013; Smith *et al*, 2013).

Although expression array-based technique has revealed novel genes associated with malignant transformation (Wrobel *et al*, 2005), this method is incapable of detecting novel gene fusions, which have been gradually recognised as potential players in cancer progression (Mitelman *et al*, 2007; Maher *et al*, 2009a).

In our study, we explored gene fusions in meningiomas using high-throughput RNA-Seq, which represented the first investigation in malignant and benign meningiomas. In one malignant sample, we identified a gene fusion event induced by local chromosomal inversion that was recently reported as a driver of neoplasia in solitary fibrous tumours (SFTs; Chmielecki *et al*, 2013; Robinson *et al*, 2013).

MATERIALS AND METHODS

The study was approved by the Institutional Review Board at the University of Southern California with informed written consent

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obtained for all participants. Patients with benign or malignant neoplasms of the brain or spine were eligible participants.

Total RNA was extracted from fresh-frozen meningioma tissue using RNeasy Kit (Qiagen, Mainz, Germany). The quality of total RNA was assessed using Experion RNA StdSens Chip (Bio-Rad, Hercules, CA, USA). TruSeq RNA Sample Prep Kits (Illumina, San Diego, CA, USA) were used for library preparation with 2 µg of total RNA as input and eight cycles of PCR amplification. The quality of libraries was assessed using High-Sensitivity DNA Chip (Agilent, Santa Clara, CA, USA). The bar-coded libraries were sequenced using the Illumina Hi-Seq 2000 platform to produce either 90-bp or 100-bp paired-end reads.

The FASTQ files were aligned to the UCSC hg19 using TopHat 2.0.4 (Trapnell *et al*, 2009). The aligned reads were processed using Cufflinks 2.0.0. The GTF annotation files for UCSC hg19 were used for gene annotation. Differential expression analysis was performed with Cuffdiff. The output results were further clustered and visualised using CummeRbund (www.compbio.mit.edu/cummeRbund/). Candidate gene fusions detected by both TopHat-Fusion (Kim and Salzberg, 2011) and Chimerascan (Maher *et al*, 2009b) were selected for analysis. All the parameters were default.

To validate fusion transcripts, 1 µg of total RNA was reversed transcribed to cDNA using M-MuLV first-strand cDNA synthesis kit (NEB, Ipswich, MA, USA) for subsequent amplification. To identify chromosomal inversion junctions, 100 ng of genomic DNA was used for PCR reactions. Primer pairs were designed using Primer3 and synthesised from Integrated DNA Technologies (IDT; San Diego, CA, USA). The KOD hot-start DNA polymerase (EMD,

Billerica, MA, USA) was used for 30 cycles of amplification. Amplified products were purified from agarose gels using Qiagen MinElute kit for Sanger sequencing.

RESULTS

Seven meningioma tissue samples (three malignant and four benign) were subject to RNA-Seq in two batches. The sequencing data indicated that the constructed libraries were mainly mRNA, with only 0.3% to 6.6% of the total mapped reads mapped to ribosomal RNA (Supplementary Table 1). Further transcriptome-based clustering presented in the dendrogram of Jensen-Shannon distances (Figure 1) showed that the four benign meningioma samples were readily separated from the malignant ones. As noted, the malignant sample 255 is separated from the other two malignant samples, confirming stronger genomic and transcriptomic heterogeneity among malignant samples.

Abnormal gene fusion events have been identified in a series of tumour types (Nowell, 1962; Tomlins *et al*, 2005; Chmielecki *et al*, 2013; Robinson *et al*, 2013). From RNA-Seq data, two gene fusions (*NAB2-STAT6* and *STAT6-NAB2*) were identified in the malignant sample 255. Interestingly, gene fusions of *NAB2-STAT6* have been reported in recurrent SFTs, including higher-grade tumours, which in the past were considered a separate entity called hemangiopericytoma (HPC; Chmielecki *et al*, 2013; Robinson *et al*, 2013). Sample 255 had an unusual histological appearance and immunophenotype with features of both meningioma and HPC (see

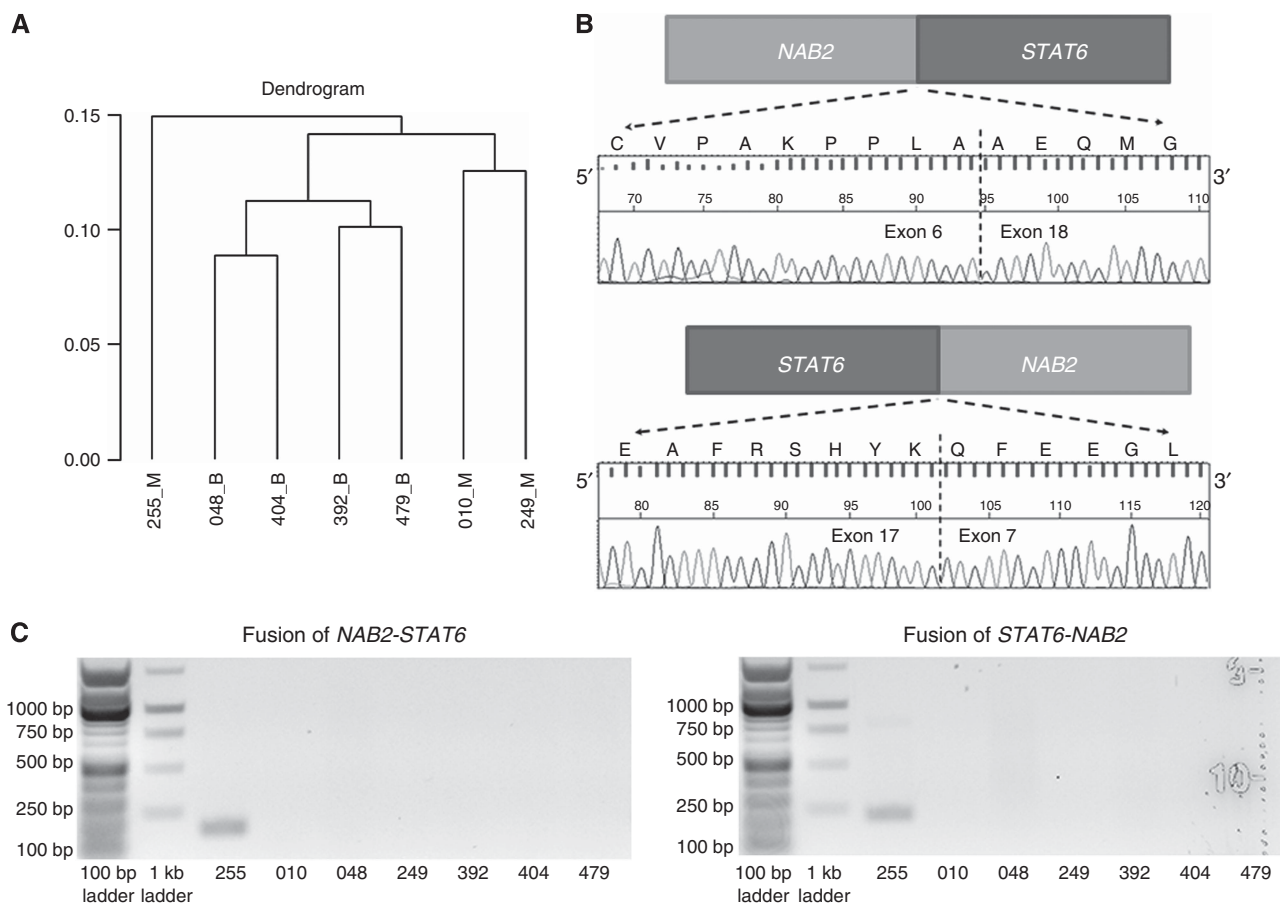


Figure 1. RNA-Seq and validation for gene fusions. **(A)** Dendrogram for clustering RNA-Seq data. Malignant and benign meningiomas were labelled with M and B, respectively. **(B)** Gene fusions of *NAB2-STAT6* and *STAT6-NAB2* validated by RT-PCR and Sanger sequencing. The translated amino-acid sequences at the in-frame fusion junctions are also shown. **(C)** Gel electrophoresis of the amplified products.

Discussion below). The fusion of *NAB2-STAT6* in our study is localised between the end of exon 6 of *NAB2* and the beginning of exon 18 of *STAT6*, with 98 junction reads identified from RNA-Seq data (Supplementary Table 2). The fusion of *STAT6-NAB2* was supported by 126 junction reads, which connects the last nucleotide of exon 17 of *STAT6* with the first nucleotide of exon 7 of *NAB2*. In other meningioma samples, these two gene fusions were not detected from RNA-Seq data (in the malignant sample 010, one junction read was detected and was likely due to noise). Interestingly, the normally spliced junctions between exon 6 and exon 7 of *NAB2* as well as between exon 17 and exon 18 of *STAT6* were also detected in sample 255, supported by 99 and 57 junction reads, respectively. Thus, the gene fusions could result from genomic alteration in one of the two alleles or a subset of the tumour cells. The gene fusion detected by RNA-Seq technique was subsequently validated using RT-PCR and Sanger sequencing. Consistent with RNA-Seq data, the gene fusions of *NAB2-STAT6* and *STAT6-NAB2* were only detected in sample 255 by RT-PCR using junction spanning primers (Supplementary Table 3). Sanger sequencing (Figure 2) validated the breakpoints of mRNA fusions detected in next-generation sequencing data. As noted, the exons of *NAB2* and *STAT6* formed in-frame fusion products. Therefore, the gene fusions occurring at the mRNA level may have resulted in novel protein products.

Both *NAB2* and *STAT6* are located on 12q13.3 (Figure 3) and are transcribed in opposite directions. Gene fusions of *NAB2-STAT6* and *STAT6-NAB2* imply potential chromosomal inversion around the q13.3 locus. Based on the gene fusion junctions identified, we hypothesise that chromosomal inversion should cover the genomic regions of exon 7 of *NAB2* as well as exons 18 to 23 of *STAT6*. Another two sets of primer pairs (Figure 3, Supplementary Table 4) were designed to detect chromosomal inversion junctions from PCR amplification of the genomic DNA. Of all seven meningioma samples used for RNA-Seq analysis, the chromosomal inversion junctions were only detected in sample 255. Additional Sanger sequencing of PCR products pinpointed the break-points at the non-coding regions of the genome.

A similar chromosomal inversion event was also reported in SFT/HPC, resulting in a novel transcript involving fusion of *NAB2* to *STAT6* and expression of the *NAB2-STAT6* fusion protein (Robinson *et al*, 2013). The transgenic Flag-tagged fusion protein binds to the promoters of *EGR1* targeting genes such as *NAB1*, *NAB2*, *TGFB1*, *CASP9* and *CEBPA* (Robinson *et al*, 2013). In the gene fusion of *NAB2-STAT6* detected in our study, the fused exons of *STAT6* encoded the transcription activation domain of *STAT6*. Interestingly, gene expression of *NAB1*, *NAB2*, *TGFB1* and *CASP9* was upregulated in sample 255 compared with other samples (Figure 3), suggesting a functional role of the gene fusion product

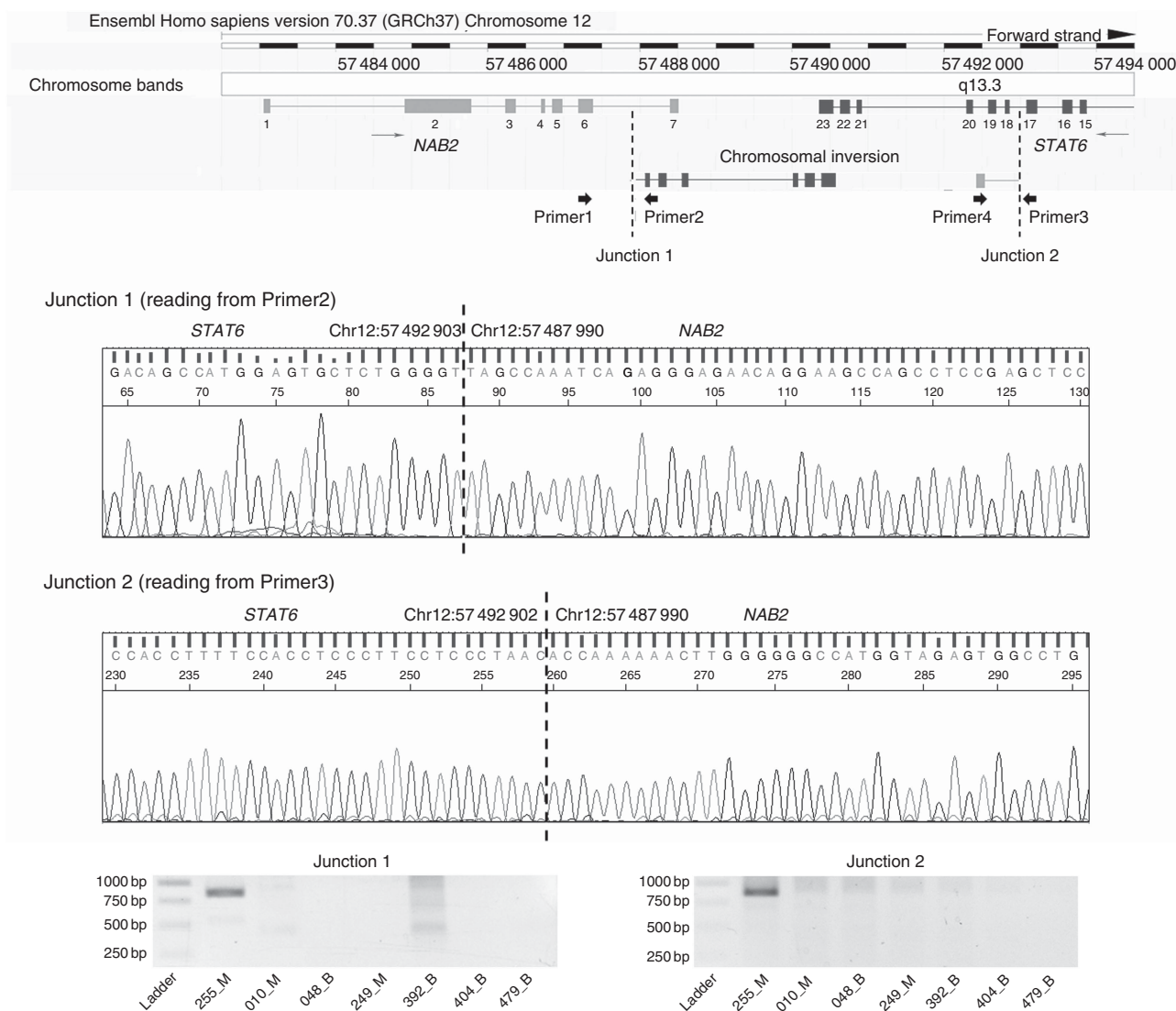


Figure 2. Chromosomal inversion around the 12q13.3 locus as identified by PCR and Sanger sequencing. The inversion break-points were highlighted in the trace maps. Gel electrophoresis demonstrates amplifications in sample 255 only.

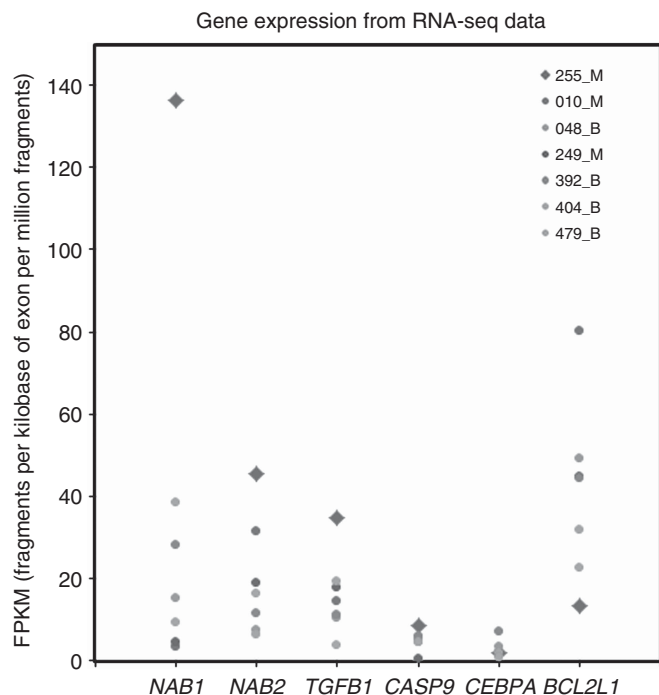


Figure 3. Expression of selected genes represented by FPKM values. Selected EGR1 targeting genes are upregulated in sample 255, whereas the STAT6 downstream target *BCL2L1* is downregulated.

in activating transcription of these genes. On the other hand, gene fusion of *STAT6-NAB2* is likely to produce a protein product incapable of transcription activation. Expression of the STAT6 downstream target gene *BCL2L1* is downregulated (Figure 3), implying a potential functional effect of *STAT6-NAB2* fusion. Thus, the gene fusions detected in our study may have roles in malignant transformation of meningiomas.

DISCUSSION

Genomic instability is a characteristic of malignant tumours. In our study, we discovered two fusion transcripts resulting from chromosomal inversion within the *NAB2-STAT6* locus. Under normal conditions, the tumour-suppressor *NAB2* regulates EGR targeting genes, whereas the transcription activator *STAT6* induces expression of *BCL2L1*. In the malignant meningioma found to harbour gene fusions, the transcription activation domain of *STAT6* fused to *NAB2* is likely to activate genes targeted by the *NAB2-STAT6* fusion protein. With loss of the activation domain, the fusion of *STAT6-NAB2* may affect downstream genes regulated by *STAT6*.

Examination of clinical information revealed that sample 255 detected with *NAB2-STAT6* and *STAT6-NAB2* fusions was the only patient sample collected after radiation treatment and/or chemotherapy. The novel gene fusions may therefore reflect the highly aggressive nature of this tumour or potentially a genomic effect of radiation and/or chemotherapy. Indeed, the DNA damage response proteins *ATM* and *ATR* were upregulated in sample 255. Therefore, we cannot exclude the possibility that radiation therapy may have induced DNA damage resulting in inversion-mediated gene fusion of *NAB2* and *STAT6*.

It is well known that it can be difficult in some instances to differentiate a SFT/HPC involving the dura from meningiomas, which can have many different histological appearances, sometimes

with features overlapping those of SFT/HPC. Fibroblastic meningioma and SFT may mimic one another. A highly atypical or malignant meningioma may lose its meningeothelial appearance and become HPC-like displaying high cellularity, a high nuclear to cytoplasmic ratio and a patternless, sarcoma-like architecture. If a tumour does not have well-developed staghorn vessels that are characteristic of HPC then it may be difficult to decide whether it is an HPC or an unusual or higher-grade meningioma. Therefore, we performed a comprehensive survey of all the pathological records for this particular tumour sample 255 with fusion.

The tumour with fusion was initially diagnosed as a 'mesenchymal neoplasm, favour meningiomas' and was first excised in 1999. As a result of its HPC-like appearance, special stains and electron microscopy were performed. The special stains that have been most widely used to differentiate meningioma and SFT/HPC are epithelial membrane antigen (EMA) and CD34. Immunohistochemistry performed on the tissue from the first excision of the tumour in 1999 was positive for both EMA and CD34, and was confirmed by recent re-staining. However, neither marker is entirely specific: typical figures are that immunoreactivity for EMA is seen in 95% of meningiomas and 20–29% of meningeal SFT/HPC, yet immunoreactivity for CD34 is seen in all SFT, 60% of HPCs, 40% of fibrous meningiomas and 60% of atypical meningiomas (Hahn *et al*, 2006). Based on electron microscopy findings, the ultrastructural features were not well preserved and were not distinctive. Although not decisive, the consensus of three pathologists who reviewed this specimen in 1999 was that it was most likely an unusual meningioma. Despite atypical features, it did not meet the WHO (1997) criteria for atypical (grade II) meningioma. No mitotic figures were identified and immunohistochemistry for Ki-67 (MIB-1) stained only about 2% of tumour cell nuclei. The tumour did not recur until 2007 but required eight additional operations over the next 5 years. Over this 5-year period, the tumour appeared increasing aggressive, eventually showing >20 mitotic figures per 10 high-power field, a WHO (2007) criterion for anaplastic or malignant meningioma. Despite relentless local progression during the 5 years, the tumour never developed distant metastasis. Five different pathologists have looked at various samples of the tumour at different times and all favour that it is a meningioma, albeit with some uncertainty.

Over the past a few years, additional molecular diagnostic markers for meningiomas have been developed. We examined a meningioma-specific biomarker Claudin-1 (gene symbol *CLDN1*, Hahn *et al*, 2006), which is absent from all SFT but present in 53%–85% of meningiomas (Bhattacharjee *et al*, 2003; Hahn *et al*, 2006). RNA-Seq data revealed active transcription of *CLDN1* in sample 255, with expression comparable to other meningioma samples (Supplementary Figure 1a). In parallel, we examined a recently generated Illumina whole-genome gene expression data set on meningiomas including sample 255, and confirmed that the expression is comparable to other meningiomas (Supplementary Figure 1b). Despite the diagnostic dilemmas occasionally encountered in differentiating meningioma from SFT/HPC, taken together, these findings lead considerable support to this patient having a meningioma that underwent malignant transformation. This raised an interesting possibility that the presence of the fusion gene imparts a more mesenchymal phenotype on a meningioma, so that it has an unusual presentation and becomes difficult to be differentiated from SFT/HPC.

A recent study (Schweizer *et al*, 2013) explored gene fusion of *NAB2-STAT6* by using strong nuclear staining of STAT6 protein as evidence for this gene fusion. Most of the 90 meningiomas showed negative, however, two tumours diagnosed as anaplastic meningioma and one as atypical meningioma were positive in immunohistochemical staining, indicating that they also carried the fusion. On review, they were found to have morphological features of both meningioma and HPC, and were recategorised as

'mesenchymal tumour not classifiable'. Although further details of radiation treatment, EMA or CD34 status were not given, similar morphological features to sample 255 in our study suggest that they may represent a distinct type of meningeal tumour.

As a single case report, it is not possible to discern any causal relationships or even statistically significant associations between a particular gene fusion and malignancy in meningiomas. Further investigation with more samples is necessary to understand the cause of genome instability observed, and whether *NAB2-STAT6* gene fusion is a cancer driver in malignant meningiomas, or simply cause a more mesenchymal phenotype.

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