Comprehensive molecular profiling of advanced/metastatic olfactory neuroblastomas

Jasmina Topcagic¹, Rebecca Feldman², Anatole Ghazalpour², Jeffrey Swensen², Zoran Gatalica², Semir Vranic¹,³,⁴*

¹ Association of Basic Medical Sciences of Federation of Bosnia and Herzegovina, Sarajevo, Bosnia and Herzegovina, ² Caris Life Sciences, Phoenix, Arizona, United States of America, ³ Department of Pathology, Clinical Center and School of Medicine, University of Sarajevo, Sarajevo, Bosnia and Herzegovina, ⁴ College of Medicine, Qatar University, Doha, Qatar

* semir.vranic@gmail.com, svranic@qu.edu.qa

Abstract

Olfactory neuroblastoma (ONB) is a rare, locally aggressive, malignant neoplasm originating in the olfactory epithelium in the nasal vault. The recurrence rate of ONB remains high and there are no specific treatment guidelines for recurrent/metastatic ONBs. This study retrospectively evaluated 23 ONB samples profiled at Caris Life Sciences (Phoenix, Arizona) using DNA sequencing (Sanger/NGS [Illumina], n = 15) and gene fusions (Archer Fusion-Plex, n = 6), whole genome RNA microarray (HumanHT-12 v4 beadChip, Illumina, n = 4), gene copy number assays (chromogenic and fluorescent in situ hybridization), and immunohistochemistry. Mutations were detected in 63% ONBs including TP53, CTNNB1, EGFR, APC, cKIT, cMET, PDGFRA, CDH1, FH, and SMAD4 genes. Twenty-one genes were overexpressed and 19 genes under-expressed by microarray assay. Some of the upregulated genes included CD24, SCG2, and IGFBP-2. None of the cases harbored copy number variations of EGFR, HER2 and cMET genes, and no gene fusions were identified. Multiple protein biomarkers of potential response or resistance to classic chemotherapy drugs were identified, such as low ERCC1 [cisplatin sensitivity in 10/12], high TOP01 [irinotecan sensitivity in 12/19], high TUBB3 [vincristine resistance in 13/14], and high MRP1 [multidrug resistance in 6/6 cases]. None of the cases (0/10) were positive for PD-L1 in tumor cells. Overexpression of pNTRK was observed in 67% (4/6) of the cases without underlying genetic alterations. Molecular alterations detected in our study (e.g., Wnt and cKIT/PDGFRα pathways) are potentially treatable using novel therapeutic approaches. Identified protein biomarkers of response or resistance to classic chemotherapy could be useful in optimizing existing chemotherapy treatment(s) in ONBs.
Introduction

Olfactory neuroblastoma (ONB), also called esthesioneuroblastoma, is a rare, locally aggressive, malignant neoplasm originating in the specialized sensory neuroepithelial olfactory cells found in the upper part of the nasal cavity [1]. Multiple modalities are currently in use to treat ONB, including surgical resection, radiotherapy, and chemotherapy. Numerous studies confirmed that a combination of surgery and radiotherapy is the treatment of choice for the majority of primary-site ONBs [2–6]. Advanced and metastatic ONBs are usually treated with classic chemotherapy, including etoposide, ifosfamide, cisplatin, cyclophosphamide, vincristine, doxorubicin, and nitrogen mustard [3,7]. However, due to the unpredictable biological behavior of the tumor and a lack of consensus on traditional treatment modalities [2,3], the recurrence rate of ONB remains high and effective treatment guidelines for high-grade ONBs are yet to be developed.

In recent years, with the advancement of molecular diagnostic methods, the focus has been on developing individualized targeted therapies for treating different types of cancer [8]. Cyto genetic studies on ONB revealed diverse and complex genomic imbalances in entire chromosomes and chromosome segments [9–11]. Several studies have reported copy number changes in ONB including gains at 7q11 and 20q and deletions at 2q, 5q, 6p, 6q, and 18q [10], as well as novel chromosome aberrations that have not been previously described. In addition, some chromosome regions could be implicated in the tumor progression and metastases formation [1,12]. These results not only indicate complex molecular processes underlying ONB, but also point to the need for a more detailed molecular characterization of ONBs at different stages of tumor progression.

Currently only a few studies have investigated genomic landscape of ONBs, using different sequencing techniques [13–17]. Furthermore, in three of these studies the potential of targeted therapy with specific drugs was explored. Weiss et al. [13] performed whole genome sequencing (WGS) on paired normal and tumor DNA from a patient with metastatic ONB. They detected mutations specific only to the metastatic ONB sample (i.e., in KDR, MYC, SIN3B, and NLRC4 genes) as well as mutations present in both, the metastatic and original surgical resection specimens (i.e., in TP53, TAOK2, and MAP4K2 genes) [1]. Analyzing cancer genomes from seven rare types of metastatic adolescent and young adult cancers (including ONB) using whole exome sequencing [WES], whole-transcriptome sequencing, or OncoScan™, Cha et al. identified TP53 missense mutation in a metastatic ONB sample, as well as a loss-of-function in CDKN2C gene [14]. Based on these results, they proposed CDK4/6 inhibitors, palbociclib and LY2835219, as potential treatment strategies [16,18]. Similarly, a recent comprehensive genomic study of Gay et al revealed alterations of TP53, PIK3CA, NF1, CDKN2A, and CDKN2C in ONBs [16]. The study of Wang et al. was first to report a case of recurrent ONB treated with a targeted therapy regimen determined after WES. Mutations in EGFR, FGFR2, KDR, and RET genes were detected, therefore the authors utilized a combination of cetuximab and sunitinib [15].

Considering the lack of standardized treatment guidelines, the potential advantages of targeted therapy approaches [8] and the paucity of data exploring the molecular pathogenesis of ONB, we explored potentially targetable biomarkers/pathways in a cohort of recurrent or metastatic ONBs, using multiplatform molecular profiling approach. We identified multiple protein biomarkers of response or resistance to classic chemotherapy and targeted therapy that could be useful in optimizing the cytotoxic chemotherapy and further improving personalized treatment of ONB.
Materials and methods

Patients and samples

This retrospective study included 23 formalin-fixed paraffin-embedded (FFPE) samples of the patients with recurrent or metastatic ONB (see S1 Excel File) profiled at the CLIA-certified laboratory, Caris Life Sciences (Phoenix, Arizona) in the period 2012–2017. Histologic diagnosis and review of results of immunohistochemical tests performed at the referring institutions to support the diagnosis of ONB were confirmed by a board certified pathologist (Z.G.) and appropriate slides were used for molecular profiling. Microdissection of tumor samples was performed when appropriate to enrich the tumor cell population.

Caris Life Sciences de-identified, remnant samples provided by participating investigators. Tumor profiling was performed and results were associated to a Subject ID. Because remnant tissue from previous samplings with no associated identifiers were utilized, this research was compliant with 45 CFR 46.101(b). Therefore, the project was deemed exempt from IRB oversight and consent requirements were waived.

Immunohistochemistry (IHC)

Expression of predictive biomarkers was evaluated immunohistochemically using commercially available antibodies and detection kits by automated staining techniques (Benchmark XT, Ventana, Tucson, AZ): antibodies against androgen receptor (AR) [n = 18], topoisomerase 1 and 2 alpha (TOPO1, TOP2A) [n = 19], estrogen receptor (ER) [n = 18], progesterone receptor (PR) [n = 18], MET proto-oncogene, receptor tyrosine kinase (c-MET) [n = 13], human epidermal growth factor receptor 2 (HER2) [n = 19], tyrosine protein c-Kit receptor kinase (c-Kit) [n = 6], epidermal growth factor receptor (EGFR) [n = 5], phosphatase and tensin homolog (PTEN) [n = 18], O(6)-methylguanine methyltransferase (MGMT) [n = 19], P-glycoprotein (PGP) [n = 16], thymidylate synthase (TS) [n = 19], transducin-like enhancer of split 3 (TLE3) [n = 12], ribonucleotide reductase M1 (RRM1) [n = 15], serum protein acidic and rich in cysteine M (SPARC-M) [n = 13], tubulin beta-3 chain (TUBB3) [n = 14], anaplastic lymphoma kinase (ALK) [n = 4], breast cancer resistance protein (BCRP) [n = 4], excision repair cross-complementation group 1 protein (ERCC1) [n = 12], multidrug resistance associated protein 1 (MRP1) [n = 6], programmed cell death-1 (PD-1) [n = 8], platelet-derived growth factor receptor (PDGFR) [n = 4], and programmed death ligand-1 (PD-L1) [n = 10], tyrosine receptor kinase (pan-antiNTRK [TrkA+B+C]) [n = 6]. Scoring system and cutoffs for all antibodies were used as described in our previous studies [19,20] (S1 Table). All IHC assays were run along with both positive and negative controls.

Copy number assays (fluorescence in situ hybridization [FISH] and chromogenic in situ hybridization [CISH])

FISH was used for evaluation of the EGFR status (Vysis LSI EGFR SpectrumOrange/CEP7 Spectrum Green Probe, Abbott) [n = 5] while HER2 [n = 11] and c-MET [n = 9] genes were evaluated using CISH (dual EGFR DNP/CEP 7 DIG probes; INFORM HER2 Dual ISH DNA Probe Cocktail; commercially available c-MET and chromosome 7 DIG probe; Ventana, Tucson, AZ) as previously described [19,21]. The tumors were considered amplified for HER2 when HER2/CEP17 ratio >2 [22]; EGFR was amplified when EGFR/CEP7 ratio >2 or >15 EGFR gene copies per cell were observed in >10% of analyzed cells [20]. cMET was amplified if >5 cMET copies on average were observed [19].
DNA sequencing (Next-generation [NGS] and Sanger sequencing)

NGS was performed on genomic DNA isolated from 15 FFPE samples using the Illumina MiSeq platform (La Jolla, CA). The Illumina TruSeq Amplicon—Cancer Panel (TSACP) was used for amplifying specific genomic regions. The NGS panel covering 46 genes were tested on 10 ONB cases while five cases were explored using the extended NGS panel that covers 592 genes (available here: http://www.carismolecularintelligence.com/solid_tumors_international) [19,21]. For selected regions of v-Raf murine sarcoma viral oncogene homolog B (BRAF), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), c-KIT, EGFR, and phosphatidylinositol 3-kinase catalytic subunit alpha (PIK3CA) genes Sanger sequencing was also used [19].

Gene fusions

Six recent ONB cases were tested for gene fusions using Archer FusionPlex Solid Tumor Kit with Illumina MiSeq (Table 1, S2 Table, and online: http://www.carismolecularintelligence.com/solid_tumors_international).

Whole genome RNA microarray

Whole-genome expression (RNA) was analyzed in four samples using Illumina cDNA-mediated annealing, selection, extension and ligation (DASL) process with the HumanHT-12 v4

Table 1. Results of in situ hybridization, sequencing and gene fusion assays.

| Case | Age (sex) | In situ hybridization | Sequencing |
|------|-----------|-----------------------|------------|
| #1   | 46 (M)    | EGFR negative         | TP53H214Y  |
| #2   | 52 (F)    | HER2, cMET negative   | c-KIT G565V; TP53 T155_V157del |
| #3   | 71 (F)    | HER2, cMET negative   | APC SNP A1474T |
| #4   | 59 (F)    | HER2, cMET negative   | w.t.       |
| #5   | 60 (M)    | HER2, cMET negative   | n/a        |
| #6   | 53 (M)    | n/a                   | w.t.       |
| #7   | 63 (M)    | n/a                   | TP53c673-1G>T |
| #8   | 51 (M)    | HER2, cMET negative   | CTNBB1 S33_H36del; [no gene fusion] |
| #9   | 52 (F)    | n/a                   | w.t.       |
| #10  | 68 (F)    | HER2, cMET negative   | w.t.       |
| #11  | 43 (F)    | HER2, cMET negative   | SMAD4 N468fs |
| #12  | 73 (F)    | n/a                   | cMET L1321I (VUS); PDGFR V546L (VUS) |
| #13  | 29 (M)    | n/a                   | n/a        |
| #14  | 50 (F)    | EGFR negative         | n/a        |
| #15  | 62 (M)    | HER2, cMET negative   | w.t.       |
| #16  | 68 (F)    | EGFR negative         | n/a        |
| #17  | 47 (M)    | n/a                   | n/a        |
| #18  | 84 (F)    | EGFR negative         | n/a        |
| #19  | 47 (M)    | HER2 negative         | CTNBB1 S33P; [no gene fusion] |
| #20  | 65 (F)    | n/a                   | w.t.; [no gene fusion] |
| #21  | 68 (F)    | n/a                   | CDH1 D756Y (VUS), FH (K477dup); [no gene fusion] |
| #22  | 59 (F)    | EGFR negative         | EGFR Q276R (VUS); [no gene fusion] |
| #23  | 37 (M)    | n/a                   | EGFR T572R (VUS); [gene fusion reaction failed] |

VUS = Variant of unknown significance; w.t. = wild type; M = Male; F = Female; n/a = not available. EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor 2; cMET: MET proto-oncogene, receptor tyrosine kinase; APC: Adenomatous polyposis coli; TP53: Tumor suppressor p53.

https://doi.org/10.1371/journal.pone.0191244.t001
beadChip (Illumina Inc., San Diego, CA) [21]. The RNA used for this analysis was extracted from FFPE samples by the Qiagen kit. The control used in this study consisted of three nerve RNAs from three healthy individuals pooled into a single sample. The over and under expression of transcripts in ONB samples were determined by taking the ratio of transcript in each ONB over the control. Transcripts were identified as significant if all the four ONB samples had consistently high or low ratio of expression when compared to control.

**Results**

**Patients**

The study included 23 patients (10 male and 13 female patients, age range: 29–84 years) with recurrent or metastatic ONB, profiled at Caris CLIA-certified laboratory in the period 2012–2017 (Table 1, S1–S3 Excel Files).

**Molecular profiling using IHC, ISH, sequencing and gene fusions**

The IHC, ISH, and sequencing results are summarized in Fig 1, Table 1 and S2 Excel File.

No cases expressed PD-L1 (0/10). Multiple protein biomarkers of response or resistance to classic chemotherapy drugs were identified: PD-1 positive tumor infiltrating lymphocytes in 25% (2/8), low ERCC1 (cisplatin sensitivity [23] in 83% (10/12), high TOPO1 (irinotecan sensitivity [24] in 63% [12/19], high TUBB3 (vincristine resistance [25] in 93% [13/14] (Fig 2F), and high MRPI (multidrug resistance) in 100% (6/6). Four out of six tested ONBs (67%) were positive for pNTRK (Fig 2E).

Mutations (pathogenic and variants of unknown significance [VUS]) were detected in 10/16 (63%) ONBs including tumor suppressor p53 (TP53) [3 cases], beta-catenin 1 gene (CTNNB1), EGFR [2 cases, respectively], while single cases harbored Adenomatous Polyposis Coli (APC), cKIT, cMET, Platelet Derived Growth Factor Receptor Alpha (PDGFR), CDH1 (E-cadherin), Fumarate Hydratase (FH) and SMAD4 gene mutations (Table 1). CTNNB1 gene alterations were further evaluated by the IHC (Fig 2C and 2D).

None of the cases harbored gene amplifications of EGFR, HER2 and cMET genes. Also, gene fusions were not identified in any of the 6 successfully tested ONBs (the panel of fusion genes is available in S2 Table).

**Microarray results**

When compared with the control tissue, 21 genes were consistently over-expressed and 19 genes consistently downregulated by an average of 10 fold. Some of the upregulated genes, such as Secretogranin II (SCG2), stem cell marker cluster of differentiation 24 [CD24] (Fig 2A), and insulin-like growth factor binding protein 2 (IGFBP-2), and downregulated genes (ATP-binding cassette transporter 8 [ABCA8] have been described to play a role in different malignancies, and were not hitherto described in ONBs (Table 2). Among the downregulated genes, GHR is a novel observation as most studies associate GHR over-expression as a risk factor for cancer. CD24 gene expression has been confirmed by the IHC (Fig 2B).

In order to better understand the pathways perturbed in ONB, we extended the list of over- and under-expressed genes to those that were consistently over- and under-expressed by two fold followed by functional classification of these genes using the Panther website (http://pantherdb.org) and the Reactome database (see S3 Excel File for the list of genes). Overall, 183 genes were found to be downregulated and 146 were over-expressed. At the nominal p-value of 0.05, fifty-nine Reactome pathways were shown to be enriched with the 183 downregulated genes in ONB including 10 genes in the organization of extra cellular matrix and 4 genes in
the cell junction organization. Pathway analysis of the 146 upregulated genes identified 100 enriched pathways including 13 genes in the Cell Cycle pathway, 10 genes in the TP53 pathway, 8 genes in Chromatin Modifying Enzyme pathway (The list of all enriched pathways can be found in S3 Excel File).

Discussion

Recent studies demonstrated potential therapeutic benefits of comprehensive molecular profiling for the patients with advanced and/or metastatic cancers [16,26–28]. In this study, we explored a wide range of potentially targetable biomarkers/pathways in recurrent or metastatic ONB samples using multiple molecular profiling platforms, including IHC, ISH, expression microarray and NGS. The sequencing results showed mutations in TP53 (n = 3/16), CTNNB1 (n = 2/16), EGFR (n = 2/16), APC, cKIT, cMET, PDGFA, CDH1, FH, and SMAD4 genes (n = 1/16, respectively). Multiple genes within the Wnt/β-catenin signaling pathway including CTNNB1, APC and CDH1 exhibited mutations within this cohort. Loss-of-function mutations in these genes lead to deregulated Wnt/β-catenin signaling and excessive stem cell renewal/proliferation, and are associated with metastatic disease [29,30]. However, we found no targetable Wnt pathway enrichment in our cohort using whole-genome expression assay. The potential of several anti-cancer drugs has been explored by targeting different stages or components of Wnt/β-catenin signaling pathway with limited success [31,32]. The role of cKIT and PDGFA mutations has been, most notably, investigated in gastrointestinal stromal tumors (GIST), where these two mutations are mutually exclusive [33]. Imatinib (tyrosine-kinase inhibitor) response in GIST patients depends not only on the protein expression, but also on the type of mutation in KIT and PDGFA genes [33,34]. One ONB case in our study had a pathogenic cKIT mutation while another harbored VUS PDGFA mutation. Both EGFR gene mutations in our study were VUS while EGFR amplification was not observed in any of the tested cases. These results indicate a limited therapeutic benefit of EGFR inhibitors in ONB patients. Mutations in TP53 gene were also detected in other studies that performed DNA sequence analysis in ONB samples [13–17]. Due to the disease progression in those patients, a role of TP53 mutation as an unfavorable prognostic and predictive factor in ONB has been suggested [18]. Of note, tumors harboring TP53 mutations may be sensitive to WEE kinase inhibitors acting against G2-M checkpoint regulators of the cell cycle WEE1 and CHK1 [35].

In addition, our microarray analysis revealed up or downregulation of several genes previously implied in carcinogenesis but not previously described in ONBs, including CD24, SCG2,
Fig 2. (A): Hematoxylin and Eosin (H&E) figure of a case with upregulation of CD24 gene by microarray, confirmed by CD24 protein overexpression in the tumor cells (B); (C) A case of olfactory neuroblastoma (ONB) with CTNNB1 mutation [S33_H36del] confirmed by the nuclear expression of β-catenin; Another case of ONB with CTNNB1 mutation [S33P] with retained cytoplasmic/membranous expression of β-catenin protein (D); A case of recurrent ONB with pNTRK overexpression (E) and overexpression of TUBB3 (F).

Table 2. Selected genes’ mRNA expression detected in olfactory neuroblastoma samples using Illumina array.

| Gene  | Location | Name/Function                                       | Relative expression ratio* |
|-------|----------|----------------------------------------------------|----------------------------|
| SCG2  | 2q36.1   | Secretogranin II/chromogranin/secrectogranin family of neuroendocrine secretory proteins | 5.6–6.7                    |
| CD24  | 6q21     | Modulates growth and differentiation of hematopoietic cells | 5.5–7.3                    |
| IGFBP-2 | 2q35 | Insulin-Like Growth Factor Binding Protein 2/promotes cell growth | 3.9–7.7                    |
| ABCA8 | 17q24.2  | Transports various molecules across extra- and intracellular membranes | 0.18–0.26                  |
| GHR   | 5p13.1   | Growth hormone receptor                             | 0.28–0.29                  |

* Compared with normal neural tissue.

Abbreviations: SCG2: Secretogranin II (member of neuroendocrine secretory proteins; the full-length protein is cleaved to produce the active peptide secretoneurin); CD24 (hematopoietic and stem cell marker); IGFBP-2: Insulin-like growth factor binding protein 2 (an oncogene in most human epithelium cancers); ABCA8 (ATP-binding cassette transporter 8); GHR: Growth hormone gene.

https://doi.org/10.1371/journal.pone.0191244.t002
and IGFBP-2 among the upregulated genes, and ABCA8 and GHR among the downregulated genes. In a recent study by Dvorak et al. a comprehensive expression analysis of all members of the ABC transporter genes across multiple cancers showed that ABCA8 downregulation was more observed in higher grade and its upregulation was associated with lower grade tumors [36]. This is consistent with the data presented in our paper that ABCA8 was consistently downregulated in all four high-grade ONB samples. Interestingly, the study by Dvorak et al. was able to show that out of all 49 ABC transporters that were investigated in various tumors, ABCA8 and four others (ABCC7, ABCC8, ABCA3, and ABCA12) were among the most dysregulated ABC genes [36]. ABCA8 has also been studied by others in relation to cancer and it has been found to be downregulated in multidrug resistant ovarian cancer cell lines [37] as well as in breast and prostate cancer [38,39]. In one study, ABCA8 was found to be upregulated in a subtype of medulloblastoma defined as Sonic hedgehog (SHH) and downregulated in the subtype defined as Wnt signaling [40]. It should be noted that the downregulation of GHR gene in ONB was an unexpected result, as most studies in the literature associate GHR upregulation with increased cancer risk [41].

More recently, immune checkpoint inhibitors (anti-PD-1/PD-L1) have revolutionized the treatment of many tumors with most remarkable benefits in the patients with melanoma, non-small cell lung carcinoma, renal cell carcinoma, bladder carcinoma, and classical Hodgkin lymphoma [42]. Our study is the first to report on the lack of PD-L1 expression in ONB samples, which makes these patients less likely to respond to anti-PD-1/PD-L1 drugs.

Recently, Gay et al. comprehensively profiled 41 samples of ONBs identifying potential targets in the mTOR, CDK and growth factor signaling pathways [16]. Other, small studies on molecular characteristics of ONB showed variable and largely inconsistent results. In the study of Weiss et al. 119 somatically lost genes and 45 gained or amplified genes were reported in a metastatic ONB sample using whole genome sequencing [13]. Seven somatic short nucleotide variants (SNVs) were validated by Sanger sequencing. Specific mutations in KDR, MYC, SIN3B, and NLRC4 genes were present only in the metastatic ONB sample, while mutations in TP53, TAOK2 and MAP4K2 genes were present in both the metastatic and original surgical resection specimens [13]. Our study confirmed some of these mutations (e.g. TP53) but failed other mutations including MYC mutation. In contrast to other genetic alterations (copy number variations, chromosomal translocations, increased enhancer activity), MYC gene mutations are uncommon [43], but have been described in some cancers (e.g. lymphomas) [44]. Furthermore, the described MYC mutation in ONB [13] has not been verified in the COSMIC database (Catalog of Somatic Mutations in Cancer). Notably, none of our ONB cases harbored MYCN gene amplification, a hallmark of pediatric neuroblastomas [45]. Further studies should definitely elucidate the role of MYC gene(s) in ONBs.

Using two different genomics platforms, Cha et al. reported a TP53 missense mutation in a metastatic ONB sample and a loss-of-function in CDKN2C gene [14]. Wang et al. detected mutations in EGFR, FGFR2, KDR, and RET genes in a recurrent ONB sample, using WES. In addition, EGFR and KDR genes were over-expressed in the tumor tissue [15].

Despite over-expression of the tropomyosin receptor kinase receptor family (NTRK) observed in 4/6 tested ONB cases, we did not detect any fusion of either one of the three NTRK genes (NTRK1, NTRK2, and NTRK3). Cancers with overexpression of NTRK driven by gene fusions had been successfully treated with novel NTRK kinase inhibitors [46] but it remains unclear if a “constitutive” overexpression such as this observed in ONB would offer any treatment advantages. Interestingly, one of the cases with NTRK overexpression exhibited TUBB3 positivity and was CD24 positive (Fig 2) indicating a potential therapeutic benefit of retinoid-based therapy [47].
ALK (anaplastic lymphoma receptor tyrosine kinase) gene alterations have been reported in various cancers including pediatric neuroblastomas [48]. We found no ALK gene alterations (mutations or fusions) or over-expression of Alk protein in any of the tested ONB cases, so these patients are unlikely to benefit from the ALK inhibitors.

In several studies, alternative approaches to ONB treatment, especially in metastatic or recurrent cases, have been explored. A combination of cetuximab (a monoclonal antibody to EGFR) and sunitinib (small-molecule inhibitor of receptor tyrosine kinases [RTKs] including kinase insert domain receptor [KDR], fibroblast growth factor receptor 2 [FGFR2], and RET RTK) was selected as a treatment regimen in a case of recurrent ONB, after WES analysis. One month after this treatment, a complete response was observed in the patient [15]. In another study, a significant improvement of clinical symptoms and disease stabilization for 15 months were observed after treatment with sunitinib in a patient with progressive ONB [49]. Furthermore, imatinib mesylate was reported as a potential second-line treatment for inducing long-term remission in heavily pretreated ONB patients [50]. Young et al. went a step further and explored the efficacy of several combinations of targeted drugs in human ONB cell line TC268 [51]. The combinations of AEW541 (insulin-like growth factor 1 [IGF-1] inhibitor) and FS114 (ribosomal protein S6 kinase beta-1 [S6K1] inhibitor) or sunitinib and FS115 (S6K1 inhibitor) were the most effective according to their results [51]. In addition, Sabongi et al. described a case with multiple recurrences of ONB adjuvantly treated with the radiolabeled-somatostatin analogue, \(^{177}\text{Lu-DOTA-TATE}\). After three cycles of \(^{177}\text{Lu-DOTA-TATE}\) treatment, the stabilization of the disease was reported [52]. Finally, Mao et al. investigated the role of SHH signaling pathway in the development of ONB, by treating ONB cell lines (TC-268 and JFEN) with cycloamine, a selective inhibitor of the SHH pathway [53]. After the cycloamine treatment, inhibited ONB cell proliferation and colony formation, induced ONB cell cycle arrest and apoptosis, downregulated expression of SHH signaling components, i.e. \(\text{PTCH1}\) and \(\text{Gli1}\), and \(\text{CCND1}\) (cyclin D1, cycle-related regulator), as well as upregulated \(\text{p21}\) expression were observed in vitro [53].

The efficacy of classic chemotherapy in ONBs remains unclear [2,4]. Chemotherapy alone, or in combination with radiotherapy, is often limited to advanced and surgically inoperable ONB cases [54]. Our IHC results indicate that several biomarkers may be used in tailoring the classical cytotoxic drugs including cisplatin and irinotecan sensitivity and vincristine resistance.

Our study have several limitations including a small sample size and lack of clinical (follow-up) data. In addition, all samples were not tested with all methodologies as these have been dynamically changing per molecular testing advances/improvements. This may result in insufficient and biased therapeutic implications.

Although our data indicate limited therapeutic options in patients with advanced and/or metastatic ONBs, several potential biomarkers that could tailor both targeted (e.g., Wnt and cKIT/PDGFRα) and classical therapeutic options merit further research. The therapeutic benefits of immune checkpoint inhibitors are less likely due to the low or lack of PD-1/PD-L1 expression.

**Supporting information**

S1 Table. The list of antibodies used for immunohistochemical biomarkers profiling. (DOCX)

S2 Table. Gene fusions tested in six olfactory neuroblastoma samples using Archer Fusion-Plex Solid Tumor Kit with Illumina MiSeq. (DOCX)
Acknowledgments

The preliminary results of the study were presented at the 40th European Society of Medical Oncology (ESMO) Congress that was held in Copenhagen, Denmark, October 7–11, 2016. We thank Yvonne Veloso, Peggy Gates, Qing Zhang, Sting Chen and Hongseok Tae (Caris Life Sciences) for their excellent technical and bioinformatics support.

Author Contributions

Conceptualization: Jasmina Topcagic, Zoran Gatalica, Semir Vranic.

Formal analysis: Rebecca Feldman, Anatole Ghazalpour, Jeffrey Swensen, Zoran Gatalica, Semir Vranic.

Investigation: Rebecca Feldman, Anatole Ghazalpour, Jeffrey Swensen, Zoran Gatalica.

Methodology: Rebecca Feldman, Anatole Ghazalpour, Jeffrey Swensen, Zoran Gatalica, Semir Vranic.

Project administration: Zoran Gatalica.

Supervision: Zoran Gatalica, Semir Vranic.

Validation: Zoran Gatalica.

Visualization: Rebecca Feldman, Anatole Ghazalpour, Jeffrey Swensen, Zoran Gatalica, Semir Vranic.

Writing – original draft: Jasmina Topcagic, Zoran Gatalica, Semir Vranic.

Writing – review & editing: Jasmina Topcagic, Rebecca Feldman, Anatole Ghazalpour, Jeffrey Swensen, Zoran Gatalica, Semir Vranic.

References

1. Thompson LD (2009) Olfactory neuroblastoma. Head Neck Pathol 3: 252–259. https://doi.org/10.1007/s12105-009-0125-2 PMID: 20596981

2. Bachar G, Goldstein DP, Shah M, Tandon A, Ringash J, Pond G, et al. (2008) Esthesioneuroblastoma: The Princess Margaret Hospital experience. Head Neck 30: 1607–1614. https://doi.org/10.1002/hed.20920 PMID: 18798301

3. Dulguerov P, Allal AS, Calcaterra TC (2001) Esthesioneuroblastoma: a meta-analysis and review. Lancet Oncol 2: 683–690. https://doi.org/10.1016/S1470-2045(01)00558-7 PMID: 11902539

4. Diaz EM Jr., Johnigan RH 3rd, Pero C, El-Naggar AK, Roberts DB, Barker JL, et al. (2005) Olfactory neuroblastoma: the 22-year experience at one comprehensive cancer center. Head Neck 27: 138–149. https://doi.org/10.1002/hed.20127 PMID: 15654688

5. Faragalla H, Weinreb L (2009) Olfactory neuroblastoma: a review and update. Adv Anat Pathol 16: 322–331. https://doi.org/10.1097/PAP.0b013e3181b544cf PMID: 19700942

6. Song CM, Won TB, Lee CH, Kim DY, Rheo CS (2012) Treatment modalities and outcomes of olfactory neuroblastoma. Laryngoscope 122: 2389–2395. https://doi.org/10.1002/lary.23641 PMID: 23070733
7. Porter AB, Bernold DM, Giannini C, Foote RL, Link MJ, Olsen KD, et al. (2008) Retrospective review of adjuvant chemotherapy for esthesioneuroblastoma. J Neurooncol 90: 201–204. https://doi.org/10.1007/s10660-008-9645-4 PMID: 18633576

8. Sethi S, Ali S, Philip PA, Sarkar FH (2013) Clinical advances in molecular biomarkers for cancer diagnosis and therapy. Int J Mol Sci 14: 14771–14784. https://doi.org/10.3390/ijms140714771 PMID: 23863689

9. Holland H, Koschny R, Krupp W, Meixensberger J, Bauer M, Kirsten H, et al. (2007) Comprehensive cytogenetic characterization of an esthesioneuroblastoma. Cancer Genet Cytogenet 173: 89–96. https://doi.org/10.1016/j.cancergen.2006.09.024 PMID: 17321323

10. Guled M, Myllykan S, Frierson HF Jr., Mills SE, Knuutila S, Stelow EB (2008) Array comparative genomic hybridization analysis of olfactory neuroblastoma. Mod Pathol 21: 770–778. https://doi.org/10.1038/modpathol.2008.57 PMID: 18408657

11. Valli R, De Bernardi F, Frattini A, Volpi L, Bignami M, Facchetti F, et al. (2015) Comparative genomic hybridization on microarray (a-CGH) in olfactory neuroblastoma: Analysis of ten cases and review of the literature. Genes Chromosomes Cancer 54: 771–775. https://doi.org/10.1002/gcc.22288 PMID: 26355525

12. Bockmuhl U, You X, Pacyna-Gengelbach M, Arps H, Draf W, Peteresen I (2004) CGH pattern of esthesioneuroblastoma and their metastases. Brain Pathol 14: 158–163. PMID: 15193028

13. Weiss GJ, Liang WS, Izatt T, Arora S, Cherni I, Raju RN, et al. (2012) Paired tumor and normal whole genome sequencing of metastatic olfactory neuroblastoma. PLoS One 7: e37029. https://doi.org/10.1371/journal.pone.0037029 PMID: 22649506

14. Cha S, Lee J, Shin JY, Kim JY, Sim SH, Keam B, et al. (2016) Clinical application of genomic profiling to find druggable targets for adolescent and young adult (AYA) cancer patients with metastasis. BMC Cancer 16: 170. https://doi.org/10.1186/s12885-016-2209-1 PMID: 26925973

15. Wang L, Ding Y, Wei L, Zhao D, Wang R, Zhang Y, et al. (2016) Recurrent Olfactory Neuroblastoma Treated With Cetuximab and Sunitinib: A Case Report. Medicine (Baltimore) 95: e3536. https://doi.org/10.1016/j.mdac.2016.02.072

16. Gay LM, Kim S, Fedorchak K, Kundranda M, Odia Y, Nangia C, et al. (2014) Comprehensive Genomic Profiling of Esthesioneuroblastoma Reveals Additional Treatment Options. Oncologist 22: 834–842. https://doi.org/10.1016/j.oncologist.2016.02-027 PMID: 28495808

17. Lazo de la Vega L, McHugh JB, Cani AK, Kunder K, Walocko FM, Liu CJ, et al. (2017) Comprehensive Molecular Profiling of Olfactory Neuroblastoma Identifies Potentially Targetable FGFR3 Amplifications. Mol Cancer Res.

18. Czapiewski P, Kunc M, Haybaeck J (2016) Genetic and molecular alterations in olfactory neuroblastoma: implications for pathogenesis, prognosis and treatment. Oncotarget 7: 52584–52596. https://doi.org/10.18632/oncotarget.9683 PMID: 27256979

19. Millis SZ, Gatalica Z, Winkler J, Vranic S, Kimbrough J, Reddy S, et al. (2015) Predictive Biomarker Profiling of > 6000 Breast Cancer Patients Shows Heterogeneity in TNBC, With Treatment Implications. Clin Breast Cancer 15: 473–481 e473. https://doi.org/10.1016/j.clbc.2015.04.008 PMID: 26051240

20. Gatalica Z, Snyder C, Maney T, Ghazalpour A, Holterman DA, Xiao N, et al. (2014) Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. Cancer Epidemiol Biomarkers Prev 23: 2965–2970. https://doi.org/10.1158/1055-9965.EPI-14-0654 PMID: 25392179

21. Gatalica Z, Vranic S, Ghazalpour A, Xiu J, Ocal IT, McGill J, et al. (2016) Multiplatform molecular profiling identifies potentially targetable biomarkers in malignant phyllodes tumors of the breast. Oncotarget 7: 1707–1716. https://doi.org/10.18632/oncotarget.6421 PMID: 26625196

22. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. (2014) Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. Arch Pathol Lab Med 138: 241–256. https://doi.org/10.5858/arpa.2013-0953-SA PMID: 24099077

23. Li S, Wu J, Chen Y, Tang W, Peng Q, Deng Y, et al. (2014) ERCC1 expression levels predict the outcome of platinum-based chemotherapies in advanced bladder cancer: a meta-analysis. Anticancer Drugs 25: 106–114. https://doi.org/10.1097.CAD.000000000000021 PMID: 24025563

24. Braun MS, Richman SD, Quirke P, Daly C, Adlard JW, Elliott F, et al. (2008) Predictive biomarkers of chemotherapy efficacy in colorectal cancer: results from the UK MRC FOCUS trial. J Clin Oncol 26: 2690–2698. https://doi.org/10.1200/JCO.2007.15.5586 PMID: 18509181

25. Hirai Y, Yoshimasu T, Oura S, Ota F, Naito K, Nishiguchi H, et al. (2011) Is class III beta-tubulin a true predictive marker of sensitivity to vinorelbine in non-small cell lung cancer? Chemosensitivity data evidence. Anticancer Res 31: 999–1005. PMID: 21496728
26. Von Hoff DD, Stephenson JJ Jr., Rosen P, Loesche DM, Borad MJ, Anthony S, et al. (2010) Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. J Clin Oncol 28: 4877–4883. https://doi.org/10.1200/JCO.2009.26.5983 PMID: 20921468

27. Silva E, Gatalica Z, Vranic S, Basu G, Reddy SK, Voss A (2015) Refractory angiosarcoma of the breast with VEGFR2 upregulation successfully treated with sunitinib. Breast J 21: 205–207. https://doi.org/10.1111/tbj.12380 PMID: 25639617

28. Birendra KC, Afzal MZ, Sochaki A, Wentland KA, Chang R, Singh S, et al. (2015) Tumor molecular profiling in the treatment of refractory cancers. J Exp Ther Oncol 11: 27–32. PMID: 26259387

29. Macdonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 17: 9–26. https://doi.org/10.1016/j.devcel.2009.06.016 PMID: 19619488

30. Dey N, Barwick BG, Moreno CS, Ordanic-Kodani M, Chen Z, Oprea-Ilies G, et al. (2013) Wnt signaling in triple negative breast cancer is associated with metastasis. BMC Cancer 13: 537. https://doi.org/10.1186/1471-2407-13-537 PMID: 24209998

31. Blagodatski A, Poteryaev D, Katanaev VL (2014) Targeting the Wnt pathways for therapies. Mol Cell Ther 2: 28. https://doi.org/10.1186/2052-8426-2-28 PMID: 26056595

32. Curtin JC, Lorenzi MV (2010) Drug discovery approaches to target Wnt signaling in cancer stem cells. Mol Cancer Ther 9: 552–566.

33. Lee JH, Kim Y, Choi JW, Kim YS (2013) Correlation of imatinib resistance with the mutational status of KIT and PDGFR A genes in gastrointestinal stromal tumors: a molecular analysis. J Gastrointestin Liver Dis 22: 413–418. PMID: 23462296

34. Hlavac V, Brynychova V, Vaclavikova R, Ehrlichova M, Vrana D, Pecha V, et al. (2013) The expression profile of ATP-binding cassette transporter genes in breast carcinoma. Pharmacogenomics 14: 515–529. https://doi.org/10.2217/pgs.13.26 PMID: 23556449

35. Ingram WJ, Crowth LM, Little EB, Freeman R, Harliwong I, Veleva D, et al. (2013) ABC transporter activity linked to radiation resistance and molecular subtype in pediatric medulloblastoma. Exp Hematol Oncol 2: 26. https://doi.org/10.1186/2162-3619-2-26 PMID: 24219920

36. Boussiositis VA (2016) Molecular and Biochemical Aspects of the PD-1 Checkpoint Pathway. N Engl J Med 375: 1767–1778. https://doi.org/10.1056/NEJMra1514296 PMID: 27806234

37. Kalkat M, De Melo J, Hickman KA, Lourenco C, Redel C, Resetca D, et al. (2017) MYC Deregulation in Primary Human Cancers. Genes (Basel) 8.

38. Boussiositis VA (2016) Molecular and Biochemical Aspects of the PD-1 Checkpoint Pathway. N Engl J Med 375: 1767–1778. https://doi.org/10.1056/NEJMra1514296 PMID: 27806234

39. Prentice J, Takahashi M, Lim I, Zhang L, Hsu E, Gao Q, et al. (2012) c-Myc regulates Notch 3 expression and cell cycle progression independently of DNA binding. Oncogene 31: 4775–4785. https://doi.org/10.1038/onc.2012.148 PMID: 22479091

40. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224: 1121–1124. PMID: 6719137

41. Amatu A, Sartore-Bianchi A, Siena S (2016) NTRK gene fusions as novel targets of cancer therapy across multiple tumour types. ESMO Open 1: e000023. https://doi.org/10.1136/esmoopen-2015-000023 PMID: 27943590
47. Shah N, Wang J, Selich-Anderson J, Graham G, Siddiqui H, Li X, et al. (2014) PBX1 is a favorable prognostic biomarker as it modulates 13-cis retinoic acid-mediated differentiation in neuroblastoma. Clin Cancer Res 20: 4400–4412. https://doi.org/10.1158/1078-0432.CCR-13-1486 PMID: 24947929

48. Caren H, Abel F, Kogner P, Martinsson T (2008) High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. Biochem J 416: 153–159. PMID: 18990089

49. Preusser M, Hutterer M, Sohm M, Koperek O, Elandt K, Dieckmann K, et al. (2010) Disease stabilization of progressive olfactory neuroblastoma (esthesioneuroblastoma) under treatment with sunitinib mesylate. J Neurooncol 97: 305–308. https://doi.org/10.1007/s11060-009-0027-x PMID: 19820899

50. Kim SA, J. R.; Bergmann L.; Ottmann O.G. (2011) Imatinib mesylate as second-line treatment in a c-kit positive esthesioneuroblastoma Journal of Clinical Oncology 29: e12513.

51. Young KA G.; Korbonits M. (2015) Nove targeted treatment combinations for malignant neuroendocrine tumour olfactory neuroblastoma. Endocrine Abstr 38: P153.

52. Sabongi JG, Goncalves MC, Alves CD, Alves J, Scapulatempo-Neto C, Moriguchi SM (2016) Lutetium 177-DOTA-TATE therapy for esthesioneuroblastoma: A case report. Exp Ther Med 12: 3078–3082. https://doi.org/10.3892/etm.2016.3732 PMID: 27882120

53. Mao L, Xia YP, Zhou YN, Dai RL, Yang X, Wang YJ, et al. (2009) Activation of sonic hedgehog signaling pathway in olfactory neuroblastoma. Oncology 77: 231–243. https://doi.org/10.1159/000236047 PMID: 19738389

54. Bhupalam LJ, S.; Laber D. (2004) Successful treatment of a patient with unresectable olfactory neuroblastoma: a case report and literature review. Journal of Clinical Oncology 22: 5620.