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Neuroendocrine Merkel Cell Carcinoma is Associated with Mutations in key DNA Repair, Epigenetic and Apoptosis Pathways: A Case-based Study using targeted Massively Parallel Sequencing

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Abstract

Merkel cell carcinoma (MCC) is a rare neuroendocrine carcinoma with poorly understood molecular etiology. We implemented a comprehensive deep sequencing approach to identify mutations in the tumor DNA from a cohort of patients treated at our institution over the past 15 years. Our results indicate mutations that may constitute therapeutic targets in MCC. Methods: Five patients were treated for MCC within the study interval. Patients with adequate tissue(N=4), positive neuroendocrine differentiation (Chromogranin, Synaptophysin, and CK20), and histopathological confirmation of MCC were included in the study. DNA was extracted from archival tumor tissue samples and analyzed by massively parallel sequencing using a targeted, multiplex PCR approach followed by semiconductor sequencing. Results: We demonstrate high penetrance nonsense mutations in PDE4DIP (N=4) as well as various missense mutations in the DNA Damage Response (PRKDC, AURKB, ERCC5, ATR, and ATRX) and epigenetic modulating enzymes (MLL3). Conclusion: We describe several mutations in potential disease-relevant genes and pathways. These targets should be evaluated in a larger cohort to determine their role in the molecular pathogenesis of MCC.

Introduction

Merkel Cell Carcinoma (MCC) is a relatively rare neuroendocrine cancer with poor prognosis that is seen with increasing frequency in the USA.[1] MCC often presents in the sixth decade of life in patients of predominantly Caucasian ethnicity. Tumors arise in UV-exposed regions of the head and neck, upper thorax and extremities.[2] The disease often presents as nonpainful nodules with local invasion in immunocompromised patients. Recently, a great deal of attention has focused on MCC due to the discovery of a viral pathogenesis for the disease.[3]

Merkel cell carcinoma is a neuroendocrine neoplasm involving somatosensory cells present within the epidermis. Merkel cells, also known as Merkel-Ranvier cells or APUD cells (apparently unrelated endocrine cells) contain neuroendocrine granules and detect coarse tactile stimuli. Their distribution occurs throughout the basal epidermis in both glabrous and haired skin including the nose, lips, and gingiva.[4] Merkel cells often juxtapose hair follicle bulges and Langerhann's cells, and are increased in mechanosensory and tactile-sensory regions. As such, these cells are innervated by A nerve fibers of the peripheral nervous system.[5] This compartment is innervated at approximately 50 cells per nerve bouton and is principally served by mechano- ( ), proprio(and C)-, and nociceptive (A and C) fibers.[6] Merkel cells are putative mechanosensory cells of the epidermis; however, the direct mechanistic role they play in neoplasia remains poorly defined at the molecular level.

MCC is considered a nonmelanotic skin cancer and has only within the past several decades gained better definition. MCC was first described by Cyril Toker as trabecular carcinoma of the skin and its diagnosis was greatly facilitated by the advent of reliable cytokeratin 20 immunohistochemical staining in the early 1990's.[7,8,9] Additionally, MCC lesions are positive for various granular neuroendocrine markers including chromogranin A and synaptophysin. Interestingly, in 2008 a DNA polyomavirus was identified and classified as the Merkel cell polyomavirus (MCPyV) that has since been detected in the majority of MCC cases.[3,10] Infection with MCPyV is believed to be associated with nearly 100% of MCC. Reactivation of latent MCPyV in immunocompromised individuals has been posited as an essential underlying pathogenic mechanism.[11]

Merkel cell carcinomas are clinically challenging to manage and often recur locally within a short time following initial resection. Management often includes broad excision followed by concurrent

chemoradiation including 5-Fluoroxyuridine (5-FU) and platinum-based regimen and there are currently no FDA-approved targeted therapies.

The management and understanding of MCC has remained limited due to the absence of deep sequencing studies to determine potential mutations within these tumors.[12,13] To this end, we have implemented a massively parallel sequencing approach covering over 400 cancer-related genes in an attempt to further dissect some of the critical oncogene drivers in a cohort of MCC patients treated at our institution.

Methods

Patient Selection

All aspects of the study were approved by the William Jennings Bryan Dorn VA Medical Center research department and institutional review board. Retrospective chart reviews conducted from 1993 to 2013 revealed a total of five patients diagnosed and treated for neuroendocrine Merkel cell carcinoma. Patient demographics, clinical metrics, history, progression free survival, and overall survival were analyzed.

Diagnostic Pathology

All cases were confirmed by a board certified pathologist for histopathological small cell differentiation as well as cytokeratin 20 (CK20), Synaptophysin (Syn), and Chromogranin A (CgrA). Additionally, Merkel cell polyoma virus (MCPyV) large T antigen was analyzed in slides from the retrospective cases and reviewed.

DNA Extraction and Massively Parallel Sequencing of 400 Cancer-related Genes.

Genomic DNA was extracted from archival formalin fixed paraffin embedded (FFPE) utilizing a FFPE DNA extraction kit as per manufacturer's recommendations (Qiagen). Briefly, tissue was sectioned at 4 μm with approximately 3 sections per block and deparaffinized in xylene. Tissue was then washed with ethanol and the sedimented samples were separated from residual solvent by evaporation. Samples were lysed in lysis buffer with 10% DNA Proteinase-K at 55 °C for 60 minutes with an additional reversal of DNA cross-links at 90 °C for 60 minutes. Lysed tissue was then added to DNA columns and submitted to a series of washes with elution in water.

AmpliSeq (Life Technologies) DNA libraries were then prepared following the manufacturer's instructions. Briefly, for each specimen, 40 ng of DNA was divided amongst four multiplex primer pools containing 10 ng of template DNA. Approximately 4000 target amplicons were amplified in each pool using the AmpliSeq Comprehensive Cancer Panel (CCP) primers and standard AmpliSeq manufacturers protocol. These amplicon pools were then combined and put through the remainder of the library preparation per the standard protocol. Libraries were diluted to 100 pM, combined in equal amounts, and used for template preparation of Ion Sphere Particles (ISPs) per the manufacturer's instructions. Prior to sequencing, the percent of templated ISPs was verified to lie between 10% and 25%, ensuring that the appropriate amount of library was added during the template preparation step. ISPs were then enriched and deposited on to a Proton PI sequencing chip per the instructions given in the Ion PI Sequencing 200 Kit user guide. Six CCP libraries were sequenced simultaneously across two Proton runs, yielding between 15.5M and 28M aligned reads per library. Alignment and Variant identification was performed using Torrent Suite version 3.6.2 using high stringency somatic detection settings.

Statistical Analysis

All statistical analysis was carried out using the Torrent Suite version 3.6.2 as described above.

Results

Patient Demographics and Clinical Course

Retrospective chart reviews conducted from 1993-2013 identified 5 patients treated at our institution and diagnosed with Merkel cell carcinoma. Patients fell within the typical diagnostic criteria with all being Caucasian males >60 years of age (Mean: 80, 61-89) (Table I). Patients were referred to the clinic following identification of characteristic MCC nodules. Interestingly, 80% (4/5) patients presented with past history of colon adenocarcinoma and actinic keratosis (Table I). One patient was incidentally diagnosed and treated with wide-band excision of the forehead. Three patients underwent direct biopsy with subsequent subtotal resection and 4/5 patients underwent salvage gross total resection. Post-operative scans demonstrated marked disease reduction. Two patients completed follow-up radiation therapy while one patient underwent chemotherapy alone. Eighty percent of

patients (4/5) presented with history of skin cancer and dermatological disorders at diagnosis (Squamous Cell Carcinoma: 2/5;40%; Melanoma: 1/5; 20%; Actinic Keratosis 4/5; 80%).

Patient Pathology

All diagnoses were confirmed by a board certified pathologist with chromogranin A (CgrA), synaptophysin (Syn), and cytokeratin 20 (Ck20) all common pathological markers of neuroendocrine Merkel cell carcinoma. All cases were positive for CgrA, Syn, and CK20 (Figure 1; Table II). Additionally, where available, tissue was stained for the surrogate marker MCPyV large T antigen which was present at the reported frequency of 75% of the cases with the most commonly used antibody (3/4 specimens) (Figure 1F).

Massively Parallel Sequencing of Patient Samples

Adequate tissue for DNA extraction was available from four archival patient specimens and the blocks were selected for further analysis utilizing the Ion AmpliSeq Comprehensive Cancer Panel (CCP) on Ion Proton instrumentation to assess somatic mutational spectra. The CCP is a multiplex PCR-based 409 gene targeted panel with rapid high-throughput assessment of mutations based on the Wellcome Trust Sanger Institute's Cancer Gene Census. Within our data set, we called an average of 4606 variants per patient (n=4; 4472-4762). One sample was found to maintain elevated variants at 12226 called variants (Supplemental Table 1; SFig. 2). This is not unexpected as this specimen was processed in higher concentration formalin had been archived for a prolonged period of time >10 years.[14,15] However, concomitant variants and deleterious mutations were detected within all genes that were interrogated within synonymous loci.

Gene Mutational Spectrum Analysis

Next-generation sequencing analysis revealed a spectrum of mutations which were present at high mutant allele frequencies (MAF). The CCP contains exon-specific probes which specifically avoid homopolymers and we corrected for the possibility of G>C and T>A transversions by focusing only on high MAFs. Due to the retrospective nature of the cohort, matched blood samples to determine somatic mutational status were unavailable. We therefore focused our analysis only on the most significant and penetrant mutations within our population.

Nonsense mutations

Several nonsense mutations were detected in 75% of patients (*n*=3/4) within the fourth exon of PDE4DIP (Table III). This gene encodes phosphodiesterase-4 interacting protein or myomegalin, a protein commonly associated with myelodysplastic disorders. Interestingly, phosphodiesterase-4D is a key interacting enzyme responsible for downstream inhibition of cyclic adenosine mono-phosphate (cAMP)-mediated activity within neural tissues.[16] Other mutations in critical DNA damage response genes (PRKDC (50%; 2/4) were also detected but occurred at lower confidence MAFs (MAF=48.25 and 2.65, respectively) (Figure 2; Supplemental Table I).

Insertion/Deletions and Missense Mutations

We next focused our analysis on single nucleotide polymorphisms (SNPs) resulting in missense mutations with a high MAF in our cohort (*n*= 45 Genes; MAF >86%). Importantly, many of these mutations are validated within functionally important coding domains based on the comprehensive cancer panel. A 1 bp indel (insT) was present in exon 14 of MLL3 at high allelic frequency across all 4 samples, resulting in a frame-shift mutation at tyrosine 816 (Fig. 2B). Because this mutation was present in all four samples, we wanted to ensure that this was not a systematic error in the sequencing data. For example, Ion Torrent sequencing is susceptible to miscalling indels within homopolymer stretches due to the difficulty in resolving signal amplitudes for multiple bases of the same type.[17] We analyzed the mutation and demonstrated that the indel identified in all four samples does not reside within a homopolymer, thus reducing the possibility that this mutation is a systematic error (S2). Furthermore, the coverage across this mutation was over 1000X in all samples, and there was no indication of strand or allelic bias. MLL3 encodes a nuclear-localized histone lysine-4 methyltransferase that is frequently deleted in myeloid leukemia and mutated in glioblastoma as well as pancreatic cancer.[18]

Additional analysis revealed consensus frame-shift mutations within a variety of genes associated with DNA damage repair and apoptosis. A missense mutation in all of the samples at position 1053 (glycine to arginine) in the ERCC5 gene associated with xeroderma pigmentosum (MAF: >99%; 3/4) and an additional missense within the same region 1080G>R (MAF: 99.13 1/4) was observed. Intriguingly, this missense mutation is present upstream of the nuclear localization sequence and represents a highly conserved residue in the enzyme. ERCC5, also known as XR-5, is a critical regulator in the nucleotide excision repair pathway (NER) whereby it removes nucleotide adducts

following chronic UV irradiation. It has been noted that MCC is most prevalent in pale-skinned UV-exposed regions of the body wherein NER is critical to maintaining genomic integrity.

We also observed a missense mutation at $298^{M>T}$ (MAF:>99%; 4/4) in the aurora kinase (AURKB) gene. Aurora kinase beta (AURKB) is a co-factor critical to maintaining mitotic-integrity that is activated in response to UV irradiation at the centromere and over-expression has been demonstrated to preclude chromosomal aneuploidy in colon cancer.[19,20] Furthermore, AURK interacts with survivin at the centromere which has recently been described as clinical target in MCC.[21] An identical mutation in the large intestine has been documented in the AURK gene and 3/4 of our patients intriguingly were treated previously for colon carcinoma (COSMIC).[22]

Misssense mutations were observed in several other DNA repair pathways including ataxia telangiectasia rad6 related (ATR; 4/4) within the ATRIP-binding domain and the associated ATRX (3/4). This is an important regulator of cell cycle progression following DNA damage and ATRIP has been demonstrated to modulate the activity of ATR in initiating DNA damage repair (Fig. 3).

Single-nucleotide polymorphism of the thyroid stimulatory hormone receptor (TSHR) were synonymously identified at position 727 $^{E>D}$ (MAF: >98%). Mutations in BCL2L2, an anti-apoptotic factor related to the BCL-2 family, demonstrated a glutamine to arginine mutation at position 133 (MAF: >97%; 4/4). Synonymous mutations in the COL1A1 ($1075^{T>A}$ MAF: >99%; 4/4) gene were also detected with high frequency.[23] Taken together, these germline mutations hold potential as novel targets that have not been previously described in MCC.

Discussion

Several well-powered studies evaluating the mutational spectrum in Merkel cell carcinoma have recently been conducted; however, these studies have not yet deeply sequenced tumors for a predicted mutational spectrum.[12,13,24] Within our focused cohort, we implemented a targeted 409-gene screen in four patients and demonstrated potential targets with relevance to Merkel cell disease. Importantly, we observed frequent nonsense mutations in PDE4DIP – better known as myomegalin – within 3/4 of the patients included in this study. Myomegalin is a predicted oncogene and gain of function mutations have been implicated in myeloproliferative disorders. Evaluation of CBC counts did not implicate aberrant mutations at the time of diagnosis suggesting a somatic mutation. All nonsense mutations occurred within the fourth coiled domain directly upstream of a breakpoint site within known PDE4DIP-PDGFRB fusion.[25]

Missense mutations within key DNA damage and apoptosis genes were also detected. H3K4 histone methyltransferase MLL3 demonstrated an insertion in our study in all the patients included in the study. This mutation adds a tyrosine residue upstream within the highly conserved plant homology domains (PHD) which flank the transactivation domain and are important in CREB-mediated gene expression.[26] MLL3 mutations have been previously demonstrated in other aggressive neuroendocrine tumors including pancreatic neoplasms as well as medulloblastomas.[27] Furthermore, MLL1 – a close analog of MLL3 and component of the methyl-tranferase complex –is essential for post-natal neurogenesis indicating a potential overlap of H3K4 methylation and over-activation in the neuroendocrine compartment.[28] Our study demonstrates a potentially novel missense mutation in MCC within the ERCC5 gene proximal to the nuclear localization sequence which was present in our cohort predominantly as a glycine to arginine substitution. In vitro work has recently linked ERCC5 signaling with ATR-ATRIP signaling in DNA damage responses to UV. In addition, UV induced damage repair pathway is known to activate aurora-kinase which interacts with downstream BCL2L2 and anti-apoptotic pathways. Therefore, it will be of great interest to determine whether loss of function at these loci is incurred with these mutations.[29] In our cohort, a glutamic to aspartic acid mutation at position 727 of the TSHR gene was present in high allelic frequency. Interestingly, recent studies have indicated this SNP, denoted as rs1991517, to be associated with enhanced risk for thyroid

cancer, DNA damage and RET-oncogene polymorphism in populations exposed to ionizing radiation.[30]

BCL2L2 is an anti-apoptotic gene of great current clinical interest. BCL2L2 is up-regulated downstream of NF -mediated pro-survival signaling and is involved in nervous tissue responses to BDNF and NGF.[31] Interactions between UV-induced DNA damage response and apoptotic pathways (ERCC5, AURK , BCL2L2) and receptor responsive elements (PDE4DIP and TSHR) could provide potential preclinical targets for validation in MCC.

Randomized clinical trials incorporating targeted therapies such as pazopanib or imatinib are underway but are hindered by the rarity of MCC, but are necessary to improve outcomes.[32] Pazopanib is a pan-receptor tyrosine kinase (RTK) inhibitor with activity in a variety of kinases including PDGFRA and FGFR3 which is under early phase clinical testing in MCC.[38,39] Various immunotherapy trials are underway evaluating the utilization of IL-12 gene therapy and Large T Antigen priming in MCC. Additionally, the potential PDGFRA-PDE4DIP fusion previously reported could also represent a potential clinical target. However, Swick and colleagues considered PDGFRA-KIT expression in a focused cohort of 23 tumors demonstrating a 95% and 65% expression of these markers, respectively, but found no activating mutations.[37] It will be of great interest to determine if the DNA damage and pro-survival pathways play a role in a larger cohort of this aggressive neuroendocrine cancer.

Conclusions

Within our small cohort of MCC cases, various genetic mutations were detected with potential relevance to MCC pathogenesis. UV exposure is a risk-factor for MCC and we describe a mutation in ERCC5, a gene which encodes a protein important in UV induced nucleotide excision repair (NER) and that is implicated in xeroderma pigmentosum.[33,34] All of the patients sequenced in this cohort demonstrated other UV-associated dermatological conditions including actinic keratosis and skin cancers.[35,36] Additionally, we found mutations in PDE4DIP, a gene which encodes a regulatory binding protein important in neural tissue associated calcium-mediated cAMP signaling. We also detected a high frequency of mutations within genes important in maintaining genomic stability including MLL3, AURK , and BCL2L2. MCC is an increasingly common diagnosis yet our center

observed 5 total cases with variable tissue fixation and increased nonsynomous variants. Therefore, further validation of these genes in larger associational cohorts and their significance as potential therapeutic targets and/or clinical prognostic indicators will better direct clinical decision and understanding of the underlying molecular drivers of Friedrich Sigmund Merkel's carcinoma.[40]

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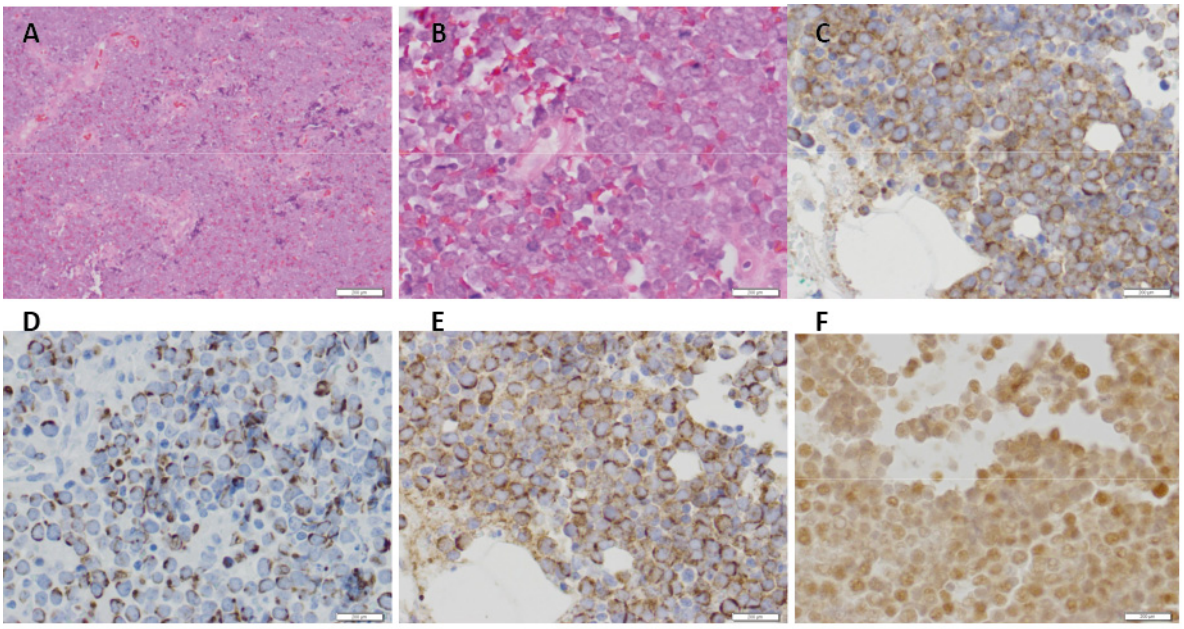

Figure 1: Representative diagnostic anatomic pathology. A) H&E 10x, B) H&E 40x, C) Synaptophysin, D) CK20, E) Chromogranin A, and F) (MCPyV Large T Antigen Immunohistochemistry. 40x unless otherwise noted; bars represent 200 microns.

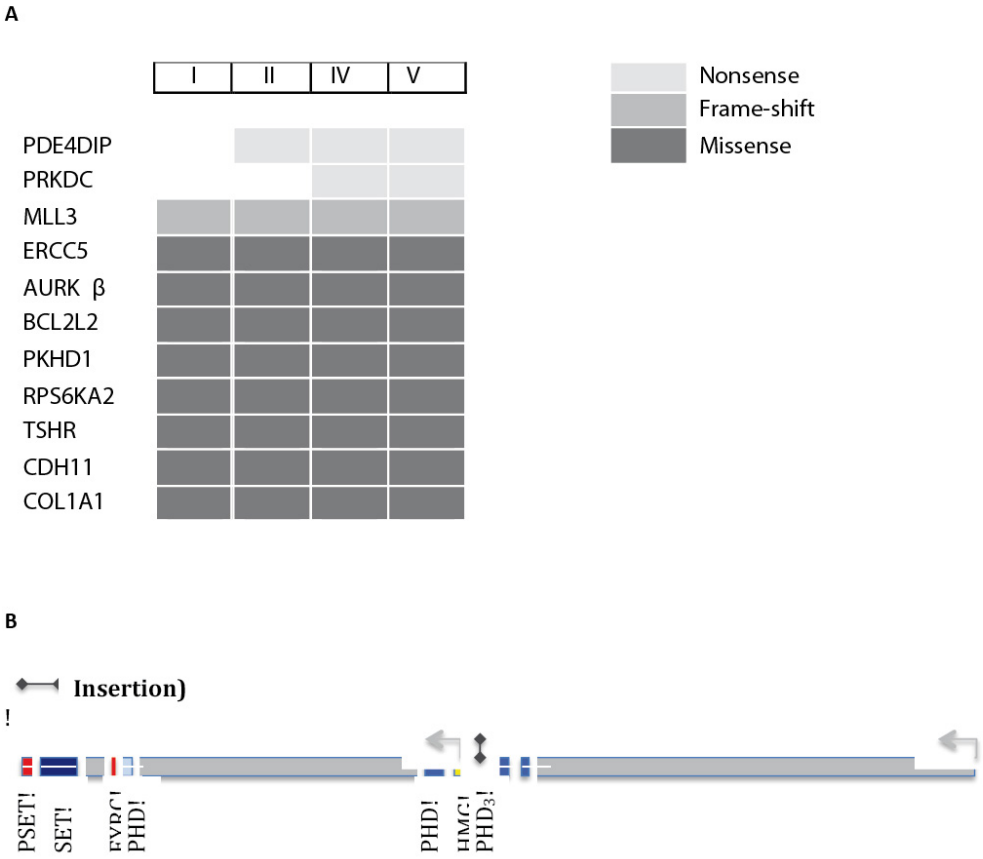

Figure 2: Mutations (MAF>86%) within the 4 sequenced samples. A) Distribution of mutations within various genes on the CCC. B) MLL3 gene with corresponding insertion in exon 14 within plant homeodomain (PHD) just up-stream of the high mobility group (HMG).

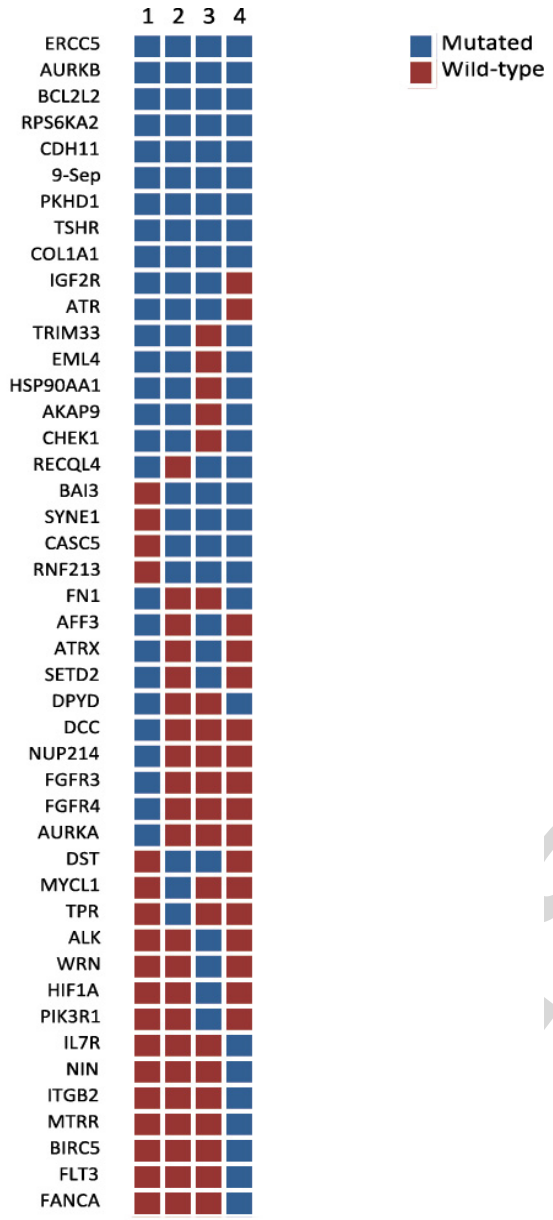

Figure 3

Figure 3: Selected missense mutations within the cohort of patients. Samples with missense mutations are noted in blue while non-mutated samples appear in red.

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