Somatic Genomic Defects And Tumor Progression In NF1-associated Tumors

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Abstract

Background: Neurofibromatosis type 1 (NF1) is a common inherited tumor predisposition syndrome. Many genetic and biochemical evidences considered *NF1* gene as a tumor suppressor gene that inactivation of both *NF1* alleles would lead to tumorigenesis. However, the clinical evaluation and early detection of malignant transformation from plexiform neurofibromas is often challenging.

Methods: We used Affymetrix's OncoScan FFPE Assay to record copy number gains and losses of common cancer genes in 24 NF1-associated tumors and compared with their *NF1* mutation status. In addition, we carried out immunohistochemical studies of NF1, SUZ12 and H3K27me3 to evaluate gene expression in NF1-associated tumors to elucidate their potential roles during neurofibromatosis.

Results: The most common Copy number alterations (CNAs) identified in this study were copy number loss (bi-allelic loss) of *NF1* (N=8) and *SUZ12* (N=7). Many *concurrent alterations of both NF1 and SUZ12* were also observed in these NF1-associated tumors. We found that the molecular events contributing to these tumors (both PNFs and MPNST) are very heterogeneous and not all MPNST harbour biallelic *NF1* inactivation. We also observed some unexplained features need to be clarified in a set of NF1-associated tumors with the genetic results and NF1, SUZ12 and H3K27me3 proteins expression.

Conclusions: We suggest that dividing NF1-associated tumors into subsets by the genetic spectrum of coordinate signaling pathway and tumor pathology may have practical implications for further choice of therapies.

Background

Neurofibromatosis type 1 (NF1) is dominantly inherited tumor predisposition syndrome that are highly variable and affect several organ system. The majority of patients with NF1 develop cutaneous neurofibromas (cNFs), and approximately one third develop benign plexiform neurofibromas (pNFs) during their lifetime. The occurrence of multiple benign dermal neurofibromas and/or plexiform neurofibromas arising from peripheral nerve sheaths, is the clinical key feature of Neurofibromatosis type 1 [1, 2]. Approximately 8–13% of NF1 patients has a lifetime risk for developing malignant peripheral nerve sheath tumors (MPNSTs) which generally arise by malignant progression of preexisting plexiform neurofibromas [3, 4].

The cause of NF1-associated tumor formation is largely explained by the underlying genetic defect of the *NF1* gene which encodes neurofibromin, a RAS-GTPase-activating protein (RAS-GAP) that is directly involved in the regulation of Ras signaling [5–8]. Many genetic and biochemical evidences considered *NF1* a tumor suppressor gene such that biallelic NF1 inactivation is the critical events in the process of NF1-associated tumorigenesis [7, 8]. In accordance with Knudson's two-hit hypothesis, somatic *NF1* inactivation has been demonstrated in both benign neurofibromas and malignant peripheral nerve sheath tumors [9], frequently evidenced by loss of heterozygosity (LOH) [10–12]. Loss of neurofibromin function may be necessary for neurofibroma formation, however, it is not sufficient for transformation into a

malignant MPNST [13]. A number of studies have attempted to identify tumor-specific molecular signatures beyond *NF1* for MPNSTs [14–19] and benign neurofibromas [17, 20]. MPNST cells harbor highly rearranged genomic architecture and have frequent loss of either CDKN2A/B or TP53 in the MPNST tumor cells, either via somatic copy number alterations (SCNAs) or single nucleotide variants (SNVs). Integrated genomic and molecular studies have later provide evidence that many chromatin modification genes may also involve in NF1 tumor progression. Loss of function mutations in the Polycomb repressive complex 2 (PRC2) component genes, such as SUZ12 or EED, are now known to be very common in MPNSTs, occurring in 23 ~ 70% of NF1-associated cases and more than 90% of sporadic cases [19, 21–23]. These PRC2 inactivation finally leads to loss of trimethylation at lysine 27 of histone H3 (H3K27me3) for regulate RAS-driven transcription [24, 25].

In this study, we present the genetic spectrum of a cohort NF1-associated tumors through a NGS targeted gene sequencing approach and a copy number analysis of *NF1* and *SUZ12* in NF1-associated tumors. Meanwhile, we have accessed tumor immunohistochemistry of NF1, SUZ12 and H3K27me3 to evaluate their essential roles during neurofibromatosis.

Methods

Study subjects and sample preparations

NF1 patients who satisfied the National Institutes of Health diagnostic criteria (NIH Consensus Statement) and who had developed either benign or malignant tumors were recruited. Tumor specimens were obtained from patients who undergo surgical resection at the Chang Gung Memorial Hospital. All patients provided informed written consent for their tissues to be used in this study, which was approved by the Chang Gung Medical Foundation Institutional Review Board. The corresponding clinical data were collected and used in accordance with proved protocols from Chang Gung Medical Foundation Institutional Review Board. Patient-matched lymphocyte DNA were also included to determine the germline event(s) of these NF1-associated tumors. Genomic DNA from archived tumor samples were prepared by PUREGENE DNA purification kit fit from GENTRA according to the manufacturer's instruction.

Targeted gene sequencing using the IT-PGM Sequencing system

A custom designed NGS panel including five common NF1-related genes: *NF1* (NM_000267, 17q11.2), *SPRED1* (NM_152594, 15q14), *KRAS* (NM_004985, 12p12.1), *BRAF* (NM_004333, 7q34), and *TP53* (NM_000546, 17p13.1) was performed for the mutation analysis in this study. The total length is 32.3 kb, 296 amplicons and the coverage is 507x. Template preparation and emulsion PCR, and Ion Sphere Particles (ISP) enrichment were performed on an Ion One Touch System by using the Ion OneTouch™ 200 Template Kit Version 2.0 (Life Technologies) according to the manufacturer's instructions. The raw PGM data were processed with Torrent Suite v3.4.2 (Life Technologies) to generate sequence reads filtered by the pipeline software quality controls. The Integrative Genomics Viewer16 (Broad Institute, Cambridge, Massachusetts) was then used to visualize variants against the reference genome and for the analysis of

depth coverage, sequence quality, and variant identification. Variant frequencies >1% in the dbSNP-Asian database were further filtered.

PCR amplification and Sanger sequencing verification

Fragments with putative *NF1* mutations or potential mutations were amplified by PCR and sequenced with Applied Biosystems 3730 Genetic Analyzer in DNA Sequencing Service of Chang Gung Memorial Hospital. A Standard Sanger sequencing was also performed exon by exon for *SUZ12*. PCR amplification were carried out under standard conditions with 30 PCR cycles and 55°-60°C annealing. PCR products will then be sequenced using the Big Dye Terminator cycle sequencing kit (Life Technologies) according to manufacturer's cycling conditions. Sequence alignments and analysis were further performed using the Autoassembler computer program (Life Technologies).

Molecular inversion probe (MIP) DNA array analysis

The Affymetrix OncoScan Assay utilizing the Molecular Inversion Probe (MIP) technology was performed for larger copy number alterations and loss of heterozygosity (LOH). This OncoScan assay contains more than 300,000 copy number and single nucleotide polymorphism (SNP) oligonucleotide probes with a median probe spacing of 4,200 kilobases (kb), with much denser coverage within known cancer genes. Twenty-four available tumor DNA samples (80 ng genomic DNA) was performed following protocols provided by manufacturer (Affymetrix, Santa Clara, CA, USA). Briefly, samples were split to separate (A/T) and (G/C) channels. After circularization, MIPs were linearized, cleaved and then amplified by PCR. Amplicons were then cleaved into two fragments (44 bp) with *HaeIII*. DNA fragments were subsequently hybridized to OncoScan® arrays at 49 °C and 60 rpm for 16-18 h. The hybridized array were then washed, stained and scanned through GeneChip® scanner 3000 7G (Affymetrix, CA). Array fluorescence intensity data (CEL files), generated by Affymetrix® GeneChip® Command Console® (AGCC, Affymetrix) Software version 1.1 were processed to produce OSCHP files and QC metrics. Samples passing QC criteria (MAPD ≤ 0.3 , ndSNPQC ≥ 26) were further analyzed through tumor Scan (TuScan) and BioDiscovery's SNPFAST2 algorithm using the Nexus Express for Oncoscan software 3.0 (Biodiscovery, Hawthorne, CA) and whole chromosome gains and losses and copy number aberrations (deletions and duplications) were determined.

Protein expression through immunohistochemistry in different NF1- associated tumors

Paraffin-embedded surgical resection specimens were retrospectively collected from the archives of the Tissue Bank at Department of Pathology, Chang Gung Memorial Hospital, for subsequent molecular analysis. Five micrometer sections mounted onto poly-L-lysine coated slides were deparaffinized and rehydrated in graded alcohols and distilled water. Endogenous peroxidase activity were quenched by incubation in 3% hydrogen peroxide in methanol for 20 min. Antigen retrieval were done by microwaving at high power for two cycles of 5 min each with a 10 min break between cycles in citrate buffer at pH 6.0. Slides were incubated with each primary antibodies at 4°C overnight. A mouse monoclonal antibody NF1 (A-12) (Sc-74445; Santa Cruz Biotechnology, Santa Cruz, CA), targeting amino acids 1-300 of the NF1

protein, was used as NF1 primary antibody. The SUZ12-antibodies used in this study was mouse monoclonal antibody against clone SUZ220A (ab126577; Abcam, Cambridge, UK). Immunohistochemistry for H3K27me3 was performed using a rabbit polyclonal antibody directed against the trimethylated lysine 27 residue of the N-terminal portion of histone H3 (1:500 dilution; Cat.# 07-449; Millipore, Billerica, MA, USA). Following the incubation of primary antibodies, these slides were then incubated with horseradish peroxidase-avidin-biotin complex (Vectastain ABC Elite, Vector Laboratories). The enzyme-binding site were visualized by 3, 3-diaminobenzidine (DAB-kit, Vector Laboratories). Non-specific binding of secondary antibody were then blocked by incubating in 10% normal horse serum in PBS containing 1% BSA. Finally, the slides were counterstained with hematoxylin and dehydrated in graded alcohol; air dried, and mounted using Resin-based mounting medium under coverslips. The results of immunoreactivity of each antibody were evaluated by pathologist and scored according to the percentage of tumor cells with positive nuclear immunostaining and staining intensity. Each sample was scored as follows: 0, negative; 1, 5% positive cells; 2, 6-50% positive cells; 3, >50% positive cells.

Statistical analysis

SPSS 13.0 software package was used for all statistical analyses. For evaluation of the difference in percentage of numbers of mutation, the Mann-Whitney U test was performed. A *P* value of <0.05 was considered to be statistical significance.

Results

NF1 mutation and copy number alterations in 24 NF1-associated tumors

A targeted gene sequencing panel with five NF1-related genes include *NF1*, *SPRED1*, *KRAS*, *BRAF* and *p53* was performed in twenty-four NF1-associated tumors from unrelated patients. In total, we found genetic alterations of *NF1* gene in 23/24 neurofibromas including 3 missense, 13 nonsense, Single splice site mutations, and 6 frameshift mutations. **Four** tumors which did not show *NF1* mutations in NGS screening were subjected to confirm for *NF1* single- and multi-exon deletions–duplications by P081-P082 MLPA analyses (Table 1). In the copy number variation analysis, we found that the most common copy number alterations (CNAs) identified in this study were copy number loss (bi-allelic loss) of *NF1* (8 of 24 tumors) and *SUZ12* (6 of 24 tumors). We have also detected the occurrence of parallel DNA alterations in these two genes (Wu-2, Wu-4, Wu-22, Wu-23, and Wu-45). The co-localization of these two genes on 17q11 might explain this coincidence of the alterations (Fig.1).

Pathology and immunohistochemistry for 24 NF1-associated tumors

A cohort of 24 NF1-associated tumors (19 neurofibromas, 2 atypical neurofibroma and 3 malignant peripheral nerve sheath tumors) from unrelated patients having a clinical diagnosis of neurofibromatosis 1 was analyzed in this study. PNFs and MPNST normally show drastic differences in histological features. Sometimes it still difficult to distinguish between the borderline tumors based on the traditional clinic pathology. **Diffuse and plexiform neurofibromas (pNFs)** are usually associated with major nerve

trunks and tortuous plexi of Schwann cells and axons embedded in stroma and show interlaced bundles of elongated cells with wavy nuclei associated with wire-like strands of collagen. MPNSTs are characterized by intersecting fascicles of monotonous spindle cells with hyperchromatic nuclei and high mitotic counts with focal areas of necrosis. Atypical neurofibromas (ANF) is later hypothesized to be a premalignant lesion and pathologically defined recently by their increased variable cellularity, cytological atypia, and more pronounced fascicular growth patterns seen in MPNST (Table 2). We then employed immunohistochemistry to evaluate NF1, SUZ12 and H3K27me3 expression levels on formalin-fixed/paraffin-embedded samples of all 24 available NF1-associated Tumors. Briefly, NF1 was strongly expressed in approximately 84.2% (16/19) of neurofibromas with a somatic or constitutional *NF1* mutation, while only weak expression in the atypical neurofibroma and malignant peripheral nerve sheath tumor. For the SUZ12, most NF1-associated tumors showed weak to moderate staining intensity (Fig 2). For the H3K27me3, most available Tumors showed heterogeneous retention of H3K27 trimethylation. Only one malignant peripheral nerve sheath tumors demonstrated complete loss of staining with the anti-H3K27me3 polyclonal antibody (Fig 3). All these results of the immunohistochemical analysis are summarized in Tables 3.

Discussion

The hallmark feature of NF1 is the occurrence of multiple benign peripheral nerve sheath tumors (neurofibromas). However, the clinical evaluation and characterization of NF1-associated tumors is often challenging. In this study, we report the utilization of Affymetrix's OncoScan FFPE Assay to record copy number gains and losses in 24 NF1-associated tumors and compared with their *NF1* mutation status. The most common Copy number alterations identified in this study were copy number loss of *NF1* and *SUZ12*. We have also observed the occurrence of parallel DNA alterations in these two genes. In addition, we carried out immunohistochemical studies of NF1, SUZ12 and H3K27me3 to evaluate gene expression to elucidate their potential roles during neurofibromatosis. We found that the molecular events contributing to these tumors (both PNFs and MPNST) are very heterogeneous and not all MPNST harbour biallelic *NF1* inactivation.

MPNSTs typically arise from a preexisting benign plexiform neurofibroma and have a poor prognosis. Accumulated evidence has suggests that *NF1* loss is necessary but not sufficient for MPNST development. In addition to *NF1* loss, MPNSTs have frequent loss of either CDKN2A or TP53 [19, 20]. CDKN2A deletions were observed in the majority of aNFs, a clear transition from a benign-atypical neurofibroma toward an intermediate-grade MPNST, by both histopathology and genetic analysis [26-30]. These mutations are rarely found in benign neurofibromas [19, 22]. A cooperative role of SUZ12 or EED inactivation, along with NF1, TP53, and CDKN2A loss-of-function, has been proposed as a key event in the development of malignancy. Recent studies have further suggested that loss of H3K27me3, which H3K27me3 represents an important epigenetic mediators of PRC2 function of chromatin regulation, may serve as a useful diagnostic marker in the distinction of malignant peripheral nerve sheath tumor from PNF [31-35]. However, we found that the molecular events contributing to these tumors (both PNFs and MPNST) are very heterogeneous and not all MPNST harbour biallelic *NF1* inactivation. We also observed

some unexplained features need to be clarified in a set of NF1-associated tumors with the genetic results and their NF1, SUZ12 and H3K27me3 proteins expression. As it has been proposed that diverse drivers of Ras-activating mutations might exist in MPNST and seem to be context dependent [19, 24, 25]. Although our sample size may not have sufficient power to detect true associations which exist among the candidate target genes with different types of neurofibromas, we believed that the validation of novel markers, identified through translational research efforts, and their incorporation into existing pathological-diagnostic system, will lead us to better refine current classification for distinct prognostic or therapeutic implications.

Conclusion

The clinical evaluation and early characterization of NF1-associated tumors is often challenging. Although PNFs and MPNST normally show drastic differences in histopathological features, sometimes it still difficult to distinguish between the borderline tumors based on the traditional pathology. We suggest that dividing NF1-associated tumors into subsets by the genetic spectrum of coordinate signaling pathway and tumor pathology may have practical implications for further choice of therapies.

Abbreviations

NF1: Neurofibromatosis type 1; FFPE: Formalin-fixed paraffin embedded; CANs: Copy number alterations; cNFs: cutaneous neurofibromas; pNFs: plexiform neurofibromas; MPNSTs: Malignant peripheral nerve sheath tumors; RAS-GAP: RAS-GTPase-activating protein; LOH: Loss of heterozygosity; SCNAs: Somatic copy number alterations; SNVs: single nucleotide variants; PRC2: Polycomb repressive complex 2; NGS: Next generation sequencing; MIP: Molecular inversion probe; DAB: 3, 3-diaminobenzidine.

Declarations

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Authors' contributions

Experimental design: YW-C. Collection and assembly of data: TH, YL, CL, CY, YW-C. Analysis and interpretation of the data: TH, CY, YW-C. Manuscript writing: CY, YW-C. Final approval of manuscript: all authors.

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Availability of data and materials

Data in this study are available upon request to the corresponding authors.

Ethics approval and consent to participate

This study was approved by the Chang Gung Medical Foundation Institutional Review Board, following the Helsinki Declaration and its later amendments. All patients provided informed written consent for their tumor specimens to be used in this study.

Consent for publication

Not applicable.

Competing interests

The authors declared no conflict of interest.

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Tables

Due to technical limitations, table 1-3 is only available as a download in the Supplemental Files section.

Figures

Figure 1

The Multi-sample comparison of different tumor samples with NF1 - SUZ12 concomitant deletions. Red, CN loss (CN<1); Blue, CN Gain (CN>2)

Figure 2

Immunohistochemical expression of SUZ12 in NF1-associated tumors. A. SUZ12 is lost in tumor cells in plexiform neurofibroma (SUZ12 loss) B. SUZ12 stain is retained in plexiform neurofibroma (no SUZ12 loss)

Figure 3

Immunohistochemical expression of H3K27me3 in NF1-associated tumors. A. neurofibroma, diffuse (+/-). B. neurofibroma, diffuse (fascicular pattern, +). C. Atypical neurofibroma (+). D. MPNST (-).

Supplementary Files

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