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High-resolution 400K oligonucleotide array comparative genomic hybridization analysis of neurofibromatosis type 1-associated cutaneous neurofibromas

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a genetic disorder where affected individuals develop benign or malignant nervous system tumors. To date, NF1 is caused by mutations in the NF1 tumor suppressor gene located at chromosome band 17q11.2. In this study, we aimed to characterize novel recurrent regional chromosomal imbalances and tumor-related candidate genes in NF1-associated cutaneous neurofibromas. Nine cutaneous neurofibromas from NF1 patients were screened for recurrent chromosomal imbalances using high-resolution 400K oligonucleotide array comparative genomic hybridization (aCGH). All the cases exhibited at least one sub-microscopic abnormality. Regions of recurrent chromosomal imbalances in a least one third of cases were loss of 1q13.2 (33%, *FAM19A3*), 1q21.1 (44%, *RABGAP1L*), 2q37.1 (56%, *INPP5D*), 3p25.1 (67%, *CHCHD4*), 4p15.32 (56%, *FGFBP1*), 5q11.2 (56%, *ARL15*), 6q22.31 (56%, *NKAIN2*), 6q22.33 (67%, *ARHGAP18*), 6q25.1 (67%, *UST*), 7q13 (56%, *ADCY1*), 12q13.13 (44%, *KRT71*), 19q13.32 (56%, *GRLF1*), and 20p11.21 (56%, *NLP*) and gain of 2p23.3 (76%, *C2orf53*), 8q22.3 (44%, *ODF1*) and 8q24.3 (67%, *ARC*). Several chromosomal imbalances, including loss of 7q11.23, 13q14.1, 14q32.13, 17p12, and 17q11.2 were detected at a lower frequency. We also confirmed that these chromosomal imbalances were not detected in the patient-matched lymphocyte DNAs. Amongst the 6 tumor-related candidate genes (*RABGAP1L*, *ADCY1*, *SLIT2*, *GRLF1*, *UST*, and *ARC*) identified in the regions of recurrent chromosomal imbalances, the gene expression changes of *UST* (down-regulation) and *ARC* (up-regulation) were found to be significantly associated with copy number alterations. The novel recurrent chromosomal imbalances and the altered expression levels of the tumor-related candidate genes may be associated with the development of NF1-associated benign cutaneous neurofibromas.

1. Introduction

Neurofibromatosis type 1 (NF1), also called von Recklinghausen disease, is an autosomal dominant genetic disorder which occurs in approximately 1 in 2500–3000 live births throughout the world (Ferner et al., 2007; Gottfried et al., 2006; Huson et al., 1989; Jouhilahti et al., 2011a; Miller et al., 2006). NF1 patients have defects in neural crest-derived tissues, leading to a wide spectrum of clinical presentations, including developmental, pigment and neoplastic aberrations (Le and Parada, 2007). The main clinical features of NF1 include café-au-lait spots (6 or more café-au-lait macules measuring at least 5 mm and 15 mm in diameter in prepubertal and postpubertal individuals respectively), freckling in the axillary or inguinal region, cognitive impairment, and neurofibromas (two or more types or plexiform neurofibroma) (National Institutes of Health Consensus Development Conference Statement Neurofibromatosis, 1988; Riccardi, 1991).

NF1-associated cutaneous neurofibromas are complex tumors composed of multiple cell types with the genotype $NF1^{+/-}$ including Schwann cells, fibroblasts, epithelial cells, and mast cells (Jouhilahti et al., 2011a). Neurofibroma is thought to be initiated by "second-hit" somatic mutation or loss of the inherited wild-type *NF1* allele in

Abbreviations: NF1, neurofibromatosis type 1; RT-PCR, reverse transcription polymerase chain reaction; BAC, bacterial artificial chromosome; CGH, comparative genomic hybridization; pNFs, plexiform neurofibromas; MPNST, malignant peripheral nerve sheath tumors; aCGH, array CGH; SNP, single-nucleotide polymorphism; LOH, loss of heterozygosity; CNN-LOH, copy number neutral-LOH; CNAs, copy number aberrations; CNVs, copy number variations; *UST*, uronyl-2-sulfotransferase; AMPARs, AMPA-type glutamate receptors; MRI, magnetic resonance imaging.

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Schwann cells, which subsequently leads to benign tumor formation, accompanied with *NF1* haploinsufficiency in other types of cells (Cichowski and Jacks, 2001). Another study found that skin-derived precursors (SKPs), a population of neural crest-like stem cells in the dermis, can transform into both plexiform and cutaneous neurofibromas through loss of *NF1* gene (Le et al., 2009). Further, a recent study reported that the development of cutaneous neurofibromas involves the recruitment of a multipotent precursor cell population with an $NF1^{+/-}$ genotype (Jouhilahti et al., 2011b). The majority of patients with NF1 develop cutaneous neurofibromas, and approximately one third develop benign plexiform neurofibromas during their lifetime. Benign tumor development, involving peripheral nerves and the central nervous system, is the hallmark feature of NF1, some of which may eventually become malignant, usually forming malignant peripheral nerve sheath tumors (MPNST) (Evans et al., 2002; Spurlock et al., 2007).

The *NF1* gene isolated by positional cloning spans over 350 kb of genomic DNA on the long arm of chromosome 17 (17q11.2) (Shen et al., 1996). The protein encoded by *NF1*, neurofibromin, has a domain homologous to the GTPase activating protein family, and downregulates Ras-GTP activity. While recent studies suggest that loss of neurofibromin is the primary genetic event for tumor initiation, additional genetic alterations are also likely required for tumor progression.

Comparative genomic hybridization (CGH) using the 32k bacterial artificial chromosome (BAC) array has been employed to identify the genetic events crucial to the development of both NF1-associated benign neurofibroma and MPNST (Mantripragada et al., 2009). While all the MPNST samples exhibited significant copy number aberrations (CNAs), the cutaneous neurofibromas did not exhibit any recurrent CNAs apart from the deletion of the 17q11 locus and reported copy number variations (CNVs) (Mantripragada et al., 2009). Furthermore, a number of MPNST-specific alterations related to malignancy were also identified using targeted microarray-based CGH (Mantripragada et al., 2008). However, to date, the molecular mechanisms underlying the pathogenesis of NF1-associated cutaneous neurofibromatosis have not been entirely resolved.

In light of the above, in the present study, we have applied the high-resolution 400K oligo-aCGH technique to analyze benign cutaneous neurofibroma from 9 NF1 patients and identified the regions of recurrent chromosomal imbalances and their representative genes involved in neurofibroma. Further, we investigated the correlation between the mRNA expression levels of the representative genes and the copy number aberrations by employing quantitative real time RT-PCR.

2. Materials and methods

2.1. Patients and tumor samples

This study was scrutinized and approved by the relevant review boards of Aichi Medical University, Japan. Prior to the initiation of the study, informed consent was obtained from the parents or guardians of all the patients to use their samples for banking and subsequent molecular analyses. The study group comprised 9 patients with primary NF1 at Aichi Medical University Hospital. The diagnoses were based on the criteria used by the National Institutes of Health (National Institutes of Health Consensus Development Conference Statement Neurofibromatosis, 1988), where at least 2 of the following 7 criteria were fulfilled: (i) 6 or more café-au-lait spots measuring at least 5 mm in diameter in prepubertal and 15 mm in postpubertal patients, (ii) multiple axillary freckles, (iii) 2 or more neurofibromas of any type or 1 plexiform neurofibroma, (iv) Lisch nodules (hamartomas), (v) optic pathway glioma, (vi) bone dysplasia, and (vii) at least 1 affected first-degree relative. In this study, fresh frozen tissue samples of benign cutaneous neurofibromas obtained from 9 patients were examined using 400K oligo-aCGH and real-time RT-PCR analysis. One tumor sample (1–2 cm in diameter) was obtained from each patient using a scalpel. The patient characteristics are summarized in Table 1. As for cutaneous tumor burden, all of the 9 patients carried low tumor counts, while the NF1 patients were classified into 2 groups depending on the tumor number as follows: (i) <5 tumors in case numbers 6, 7, and 9 and (ii) <50 tumors in case numbers 1, 2, 3, 4, 5, and 8.

Table 1
Clinical features of the 9 neurofibromatosis 1 patients.

	Case no.								
	1	2	3	4	5	6	7	8	9
Age	16	46	35	33	30	65	35	51	33
Sex	F	F	F	F	F	M	M	M	F
Family history	−	ND	ND	−	+	+	−	+	−
Café au lait spots	+	+	+	+	+	+	+	+	+
Cutaneous neurofibromas	+	+	+	+	+	+	+	+	+
Plexiform neurofibromas	+	−	−	ND	−	−	ND	−	−
Lisch nodules	−	−	−	−	−	−	ND	−	−
Freckling in the axillary or inguinal region	+	+	+	+	+	+	+	+	+
Osseous lesion	−	−	−	−	−	−	−	−	−
Spinal neurofibromas	−	+	−	+	−	+	ND	−	−
Intracranial processes[a]	+	−	−	ND	−	−	ND	−	−

ND, not done.
[a] Intracranial processes, including hamartomatous lesions and/or optic gliomas, were detected using MRI scans.

2.1.1. Isolation of genomic DNA and total RNA

For oligo-aCGH, genomic DNA was obtained from both tumor samples and peripheral blood of 9 NF1 patients using proteinase K/RNAse treatment and phenol–chloroform extraction according to standard procedures. Total RNA was also extracted from all the patient samples using Trizol reagent (Life Technologies, Carlsbad, California, USA) by guanidinium thiocyanate–phenol–chloroform extraction method.

2.2. Oligo-array CGH (aCGH)

Oligo-aCGH was performed by using the Agilent Human Genome CGH Microarray Kit 2 × 400K (Agilent Technologies, Santa Clara, California, USA). This array contains approximately 400,000 60-mer oligonucleotide probes. Labeling and hybridization were performed according to the manufacturer's protocol. The peripheral blood of 8 healthy volunteers was used as reference DNA. Briefly, 2 μg each of patient's DNA and reference DNA samples were double digested with *AluI* and *RsaI* (Promega, Madison, Wisconsin, USA) and purified with the QIAprep Spin Miniprep kit (QIAGEN K.K., Tokyo, Japan). Subsequently, the patient's DNA and reference DNA samples were labeled by random priming with either, Cy3-dUTP or Cy5-dUTP using the Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies). Following the labeling reaction, the individually labeled patient's DNA and reference DNA samples were pooled and concentrated using an Amicon Ultracel 30K device (Millipore, Bilerica, Massachusetts, USA). Then, the microarrays were hybridized using the Sure Hyb chamber (Agilent Technologies) in a rotisserie oven at 65 °C for 40 h. The hybridization mixture comprised the labeled DNAs, 2× Hybridization buffer (Agilent Technologies), 10× blocking agent (Agilent Technologies), and human Cot-1 DNA (Invitrogen). Four post-hybridization washes were carried out using the oligo-CGH washes kit (Agilent Technologies): wash buffer 1 at room temperature for 5 min, wash buffer 2 at 37 °C for 1 min, an acetonitrile rinse at room temperature for 1 min, followed by stabilization and drying solution at room temperature for 30 s. The arrays were scanned using a DNA Microarray Scanner (Agilent Technologies), and the spot intensities were measured using the Feature Extraction Software (version 11.0.1.1, Agilent Technologies). Analysis and visualization were performed with CGH Analytics software (version 4.081, Agilent Technologies), using the statistical algorithm ADM-1 and the common aberration detection method 2 with sensitivity thresholds at 2.0 and 6.0, respectively, and a moving average window of 0.2 Mb (Sung et al., 2011). Somatic genomic alterations were detected as follows: (i) loss