Chromosomal imbalances in malignant peripheral nerve sheath tumor detected by metaphase and microarray comparative genomic hybridization

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Abstract. Malignant peripheral nerve sheath tumors (MPNSTs) are highly malignant tumors affecting adolescents and adults. There have been a few reports on chromosomal aberrations of MPNSTs; however, the tumor-specific alteration remains unknown. We characterized the genomic alterations in 8 MPNSTs and 8 schwannomas by metaphase comparative genomic hybridization (CGH). In 5 of 8 MPNSTs, microarray CGH was added for more detailed analyses. Frequent gains were identified on 3q13-26, 5p13-14, and 12q11-23 and frequent losses were at 1p31, 10p, 11q24-qter, 16, and 17. Microarray CGH revealed frequent gains of EGFR, DAB2, MSH2, KCNK12, DDX15, CDK6, and LAMA3, and losses of CDH1, GLTSCR2, EGR1, CTSB, GATA3, and SULT2A1. These genes seem to be responsible for developing MPNSTs. The concordance rate between metaphase CGH and microarray CGH was 66%. Metaphase CGH was useful for identifying chromosomal alterations before applying microarray CGH.

Introduction

The malignant peripheral nerve sheath tumor (MPNST) is a high-grade soft tissue sarcoma which accounts for 5-10% of all soft tissue sarcomas. It frequently occurs in adolescents and young adults (1). MPNSTs are derived from two different backgrounds; 50% of cases have a hereditary neurofibromatosis type 1 (NF1) and others are sporadic. In the formation of neurofibromatosis, germ-line mutations occurring in genes, NF1 (17q11.2) and NF2 (22q), play an important role (2). MPNSTs develop in 10-14% of NF1 patients (3). Most patients with MPNST have previously had a benign peripheral nerve sheath tumor (BPNST) (1). However, the mechanism of malignant change from BPNST to MPNST is unknown. In recent years, there have been a few reports on chromosomal investigations of MPNSTs; CGH analyses revealed several characteristic chromosomal imbalances in a limited number of cases. NF1 (17q), NF2 (22q), p16 (9p), and EGFR (7p) alterations are reported to be involved in MPNST formation (4).

In 1998, microarray CGH technique was developed by Pinkel D *et al* (5) after modification of the metaphase CGH technique which was developed by Kallioniemi (6). Precise identification of each gene with copy number changes has become possible using this technique; however, there have been few reports on microarray CGH analyses in bone and soft tissue sarcomas. In the current study, we analyzed the chromosomal aberrations with metaphase CGH to detect the characteristic alterations in MPNSTs and to estimate the difference of chromosomal alterations between MPNSTs and schwannomas. Moreover, the aberrations detected by metaphase CGH were further analyzed by microarray CGH in 5 MPNSTs. The results of both techniques were compared in order to clarify the usefulness of both forms of CGH in chromosomal analyses.

Materials and methods

Tumor samples. Sixteen tumor samples were obtained by open biopsy or surgical excision at Okayama University Hospital between 1980 and 2003. These samples were preserved in a deep freezer at -80˚C. Among them, 8 MPNSTs from 8 patients and 8 schwannomas from 8 patients, with abundant stock samples and from which DNA could be extracted, were selected for cytogenetic analysis for this study. The patient characteristics were as matched as possible, including age, gender, tumor location, and size, in both groups.

In the 8 MPNSTs, 5 tumors were primary and 3 were locally recurrent. None of the tumors had received chemotherapy or radiotherapy between relapse and excision. Four tumors were derived from 4 females and 4 from 4 males. The average age at diagnosis was 39 years (range, 11-75 years).

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Key words: chromosomal imbalances, nerve sheath tumor, microarray, genomic hybridization

Table I. Clinico-pathological data of malignant peripheral nerve sheath tumors and schwannomas.

Case	Diagnosis	Sex	Age (years)	*NFI* mutation	Site	Surgical margin[a]	Period before relapse (months)	Relapse pattern	Period of survival (months)	Prognosis
1	MPNST	F	75	negative	thigh	intralesional	0	local and metastasis	15	DOD
2	MPNST	F	34	positive	buttock	intralesional	3	local	4	DOD
3	MPNST	M	41	negative	thigh	wide	19	local	48	DOA
4	MPNST	M	26	positive	upper arm	marginal	31	multiple	51	DOD
5	MPNST	F	53	negative	thigh	wide	11	metastasis	22	DOD
6	MPNST	F	26	positive	thigh	wide	15	multiple	21	DOD
7	MPNST	M	11	positive	knee	marginal	11	local and metastasis	25	DOD
8	MPNST	M	49	positive	back	wide	13	local	29	NED
9	scwannoma	M	60	negative	back	marginal	no relapse	none	96	CDF
10	scwannoma	F	69	negative	thigh	marginal	no relapse	none	78	CDF
11	scwannoma	F	24	negative	hand	marginal	no relapse	none	48	CDF
12	scwannoma	F	24	negative	lower leg	marginal	no relapse	none	36	CDF
13	scwannoma	F	48	negative	thigh	marginal	no relapse	none	33	CDF
14	scwannoma	M	38	negative	shoulder	marginal	no relapse	none	33	CDF
15	scwannoma	M	57	negative	thigh	marginal	no relapse	none	26	CDF
16	scwannoma	M	61	negative	thigh	marginal	no relapse	none	20	CDF

[a]According to Enneking *et al* (7); MPNST, malignant nerve sheath tumor; DOD, dead of disease; DOA, dead of another cause; CDF, continuous disease free; AWD, alive with disease.

Five patients had von Recklinghausen disease (neurofibromas type 1). All MPNSTs were high-grade tumors (stage IIB) and the surgical margins were composed of 4 wide, 2 marginal and 2 intralesional, according to the methods of Enneking *et al* (7). Six tumors were located in the extremities and 2 tumors were located in the trunk. Table I demonstrates the clinical and pathological data of the patients with MPNST. One patient (case 6) received chemotherapy of MAID regimen, composed of doxorubicin, ifosphamide, and dacarbazine (8), after the primary tumor excision. One patient (case 4) received a 40-gray of postoperative radiotherapy to another lesion in a different site from the primary tumor site.

All 8 schwannomas were primary and sporadic tumors. Four tumors were from 4 women and 4 from 4 men. The average age at diagnosis was 48 years (range, 24-69 years). Six tumors were located in the extremities and 2 tumors were located in the trunk. Every patient underwent marginal excision of the tumor following confirmation of the histological diagnosis by a rapid frozen section at an open biopsy. The clinical and pathological data of the patients with schwannoma appear in Table I.

The period of follow-up ranged from 8 to 84 months (average, 31.5 months). If the MPNSTs newly developed in the nerve of the different site during follow-up, they were regarded as multiple tumor occurrences. If the tumor appeared in the region adjacent to the previous operative site, the tumor was regarded as a locally relapsed tumor. If the tumor occurred in the area not related to the nerve or previous operation, the tumor was classified as a metastatic tumor. The end-point of the follow-up was August 2005.

Metaphase CGH. Reference DNA from healthy blood donors (male) and tumor DNA from fresh frozen tumor tissues were labeled by the nick translation method with SpectrumRed-dUTP (Vysis, IL, USA) and SpectrumGreen-dUTP (Vysis), respectively. Nick translation and CGH hybridization were performed according to the manufacturer's protocol (Vysis). Normal lymphocyte metaphase preparations were denatured at 73˚C for 5 min in 70% formamide/2X SSC (pH7) and dehydrated. The probe mixture, after ethanol precipitation and re-suspension in 10 μl CGH buffer (Vysis), was denatured at 75˚C for 5 min, applied to the slides and hybridized for 3 days at 37˚C.

The hybridization was analyzed using a Leica microscope (DMRA2) (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and the Leica Cytogenetic Workstation (CW4000) (Leica Microsystems Imaging LTD, Cambridge, UK) based on a high-sensitivity interrating CCD camera (SenSys0401E, Roper Scientific Germany, Ottobrunn, Germany) and an automated CGH analysis software package. Ratio profiles were averaged from 10 metaphases per sample. Gains of DNA sequences were defined as chromosomal regions with a

Table II. Copy number changes in 8 malignant peripheral nerve sheath tumors (MPNSTs) and 8 schwannomas.

Diagnosis	Case	Gain	Loss
MPNST	1	6q12-q22, **7q**, 11p, **12**, 13q22-qter, 18, 19, 22q	8p, 10p, 11q23-qter, 16
	2[a]	**2**, 3q24-q28, 4q, 6p, **7p**, 7q11.2-q22, 8q13-qter/**8q13-q23**, 9q31-qter/**9q34**, 12p11.2-p12, 14q22-q24, 15q22, 17q24, 18q22-qter, 21q21	6q27, 9q12, 16q12-q22, 19q, 20q
	3	1p22-p31, 4p15-q27, 5p14-q22, **6q**, 9q21-q31, 11p22-p15, 11q14-q22, 14q, 15q, 18q, 21q	1q41-qter, 2qter, 16q
	4[a]	1p31-q31, 2, 3, 4q32-pter, 5p, 5q21-q31, 6/**6q**, 7p21, 8q/**8q21-q22**, 9q21-q32, 11p, 12p12, 12q21, 14q12-q24, 15q21, 18q12-qter, 20p, 21q21	1p34-pter, 10p, 17pter, 19p
	5	1p12-p31, 1q24-q32, 2q32, 3q24-q26, 4, 5, 6q12-q23, 7q21-q31, 9p21-pter 10q21, 11p14, 11q22, 12q21, 13q12-q32, 14q12-q24, 15q12-q22, 18q12-qter, 21q21	19p
	6[a]	4p15-q27, 5p15-q31, 6p12-qter, 11p/**11p14**, 11q12-q22/**11q14-q22**, 12q12-q21, 15q11-q23, 17q23	1q32-qter
	7[a]	1p31, 1q/**q23**, 2q22-q32, **3**, 4, 5p, 6q12-q22/**6q14**, 7/**7pter-q23**, 8/**8p12-q23**, 9q21-q22, 10p13-q21, 11p14, 11q14, 12p12-q23, 13q12-q22, **14q**, 15q21, 18q, **21q**, 22q21	11q12, 11qter, 17pter, 17qter
	8[a]	1q32-qter, 2q23-q32, 3p12-q13, 3q24-q26, 4q26-q28, 5q21-q23, 6q14-q22, 8q12-q22, 9p, 10q22-q23, 11q22-q23, 13q22, 21q12	1p33-pter, 9q34, 16p, 20p12, 20q13, 22q
schwannoma	9		9p, 10p, 22q
	10	1q31	12qter, 16p, 19q13, 22q
	11	21q22	4q34, 8p, 9p, 22q
	12		22q
	13		
	14		
	15		
	16		8p, 20q, 22q

High-level gains are indicated in bold. [a]Patients with von Recklinghausen's disease.

fluorescence ratio >1.25 and losses as regions with a ratio <0.75. A positive control with known aberrations and a negative control were included in each CGH experiment as quality controls. Regional shifts of the fluorescence ratio profile exceeding the 1.5 threshold were rated as high-level gain. Telomeric and heterochromatic regions near the centromeres and the entire X and Y chromosomes were excluded from the analysis. The profiles of 1p32-pter, 16p, 17p, and chromosomes 19 and 22 were carefully investigated because they are known to give false-positive results. Judgement was based on a consensus of at least two of three authors in all cases without reference to the patient's clinical information.

Microarray CGH. Preparation of the array, hybridization, and analysis were followed according to the methods of Bruder *et al* (9). Reference DNA and test tumor DNA samples were labeled using the Random Priming Reagent kit (Vysis). Sample DNA was labeled with 1 mM Cy™ 3-dCTP (Perkin-Elmer, Boston, USA). This was mixed with whole genomic reference DNA that was labeled with Cy™ 5-dCTP (Perkin-Elmer). Both labeled DNA were co-hybridized to a microarray in the presence of human Cot 1 DNA. The microarray contained 287 kinds of target clone DNA (P1, PAC or BAC clones) representing regions that are important in cytogenetics and oncology, including oncogenes, tumor suppressor genes and subtelomere (GenoSensor® Array 300; Vysis). DNA clones comprising the desired target sequences were arrayed in target spots of approximately 75-125 μm diameter, whereby each clone is represented by 3 targets spots. Following hybridization, target spots are counter-stained. Arrays were imaged using the GenoSensor and the GenoSensor Reader software. The GenoSensor Reader System is a large-field multicolor fluorescence imaging system which captures an image of the hybridized chip in 3 color planes; Cy3, Cy5, and DAPI blue. The included software automatically identifies each spot and, by analysis of the set of Cy3/Cy5 ratios on all targets, calculates the ratio most representative of the modal DNA copy number of the sample DNA. The normal range of ration of control DNA was defined between 1.14 and 0.86. If the data was beyond the range of +1 and -0.15, it was regarded as gain and loss, respectively.

Results

The clinical courses of the patients. Six of 8 patients with MPNST died from their disease, 1 patient died from another

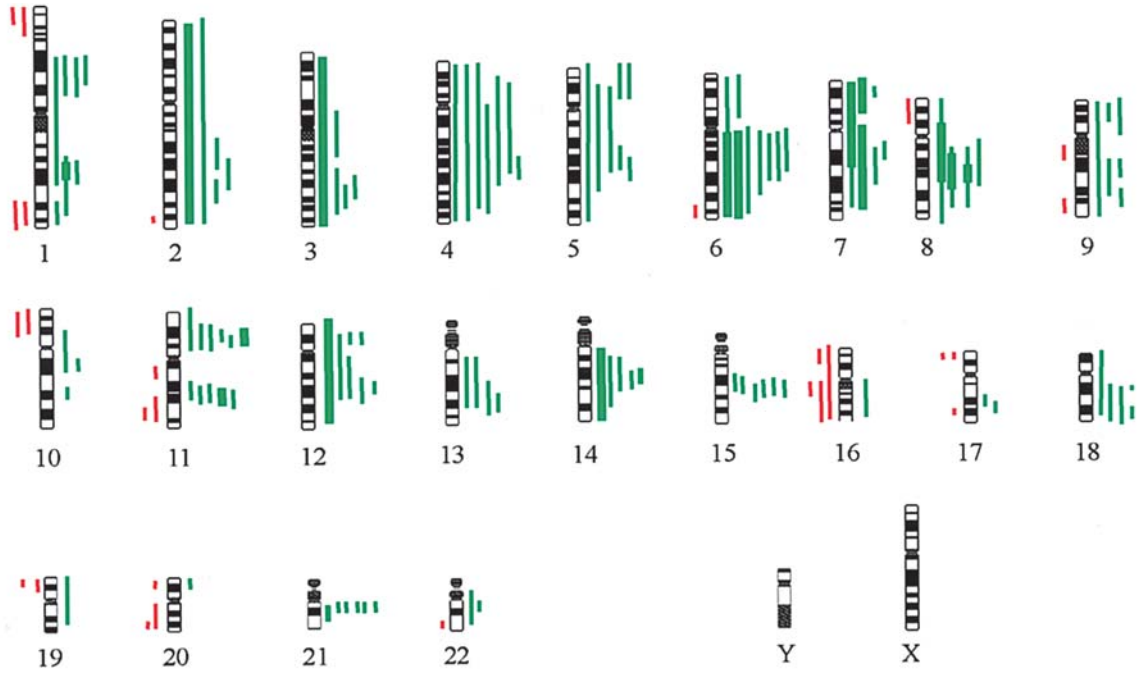

Figure 1. Summary of gains and losses of DNA sequence copy number in MPNST samples analyzed by CGH. Losses are shown on the left side and gains on the right. Each line represents a genetic aberration seen in one sample. High-level gains are shown as thick lines.

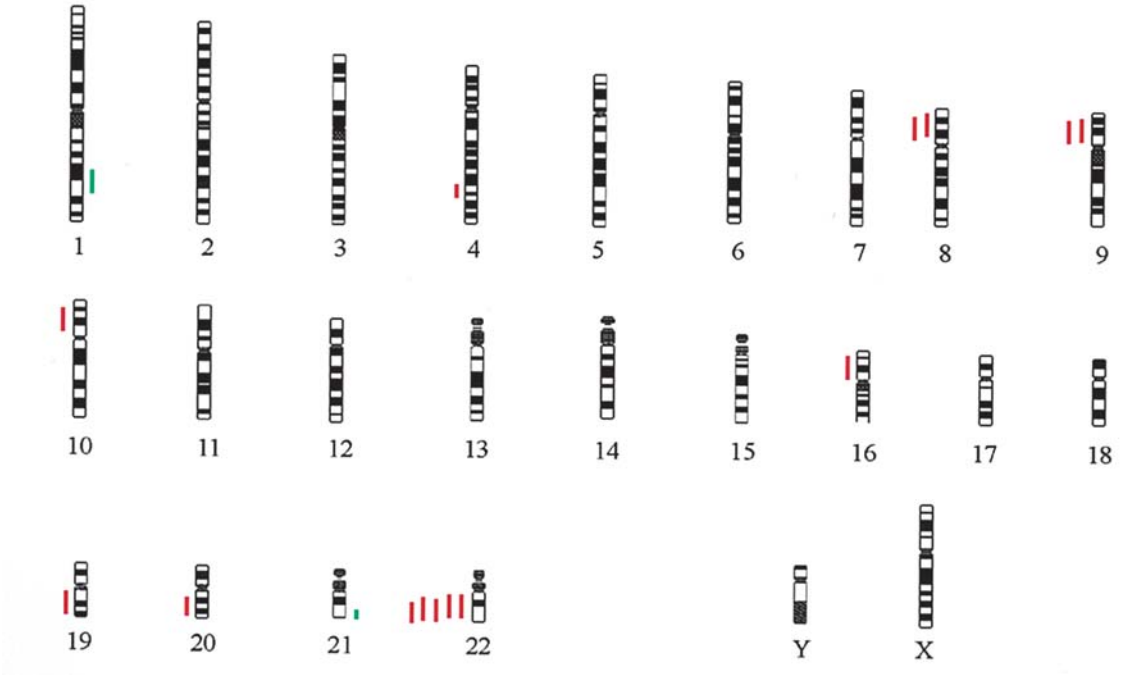

Figure 2. Summary of gains and losses of DNA sequence copy number in schwannoma. Losses are shown on the left side and gains on the right. Each line represents a genetic aberration seen in one sample.

cause (suicide), and 1 patient is still alive (Table I). Three patients had a local recurrence (cases 2, 3, and 8), 1 patient had a pulmonary metastasis (case 5), 2 patients had both a local recurrence and a pulmonary metastasis (cases 1 and 7), and 2 patients had multiple tumor occurrences during the follow-up (cases 4 and 6). The average disease-free and survival periods were 15 months (range 3-31 months) and 27 months (range 4-51 months), respectively.

All patients with schwannoma are alive and free from local recurrence or metastasis. The average disease-free period was 46 months (range 20-96 months).

Metaphase CGH. Fresh-frozen tissue specimens from 8 MPNSTs and 8 schwannomas obtained from 16 patients were analyzed by metaphase CGH. Table II demonstrates the copy number changes in MPNSTs and schwannomas

Table III. Results of microarray CGH in 5 malignant peripheral nerve sheath tumors.

Target index	Name of DNA clone	Location	Case number				
			1	2	3	4	8
24	**U32389**	2p tel	**1.16**	**1.33**	0.97	**1.48**	**1.41**
25	**2PTEL27**	2p tel	**1.25**	**1.31**	0.91	**1.19**	**1.46**
27	**MSH2,KCNK12**	2p22.3-2p22.1	**1.31**	**1.59**	1.05	**1.2**	**1.28**
60	**DDX15**	4p15.3	**1.19**	**1.29**	1.08	**1.28**	**1.4**
71	**DAB2**	5p13	**1.26**	**1.44**	**1.21**	**1.34**	**1.19**
74	*EGR1*	5q31.1	*0.82*	*0.81*	0.89	*0.82*	*0.85*
91	**EGFR**	7p12.3-p12.1	**1.86**	**1.94**	**1.26**	**1.22**	**1.55**
95	**CDK6**	7q21-q22	**1.79**	**1.2**	**1.17**	0.99	**1.29**
103	*CTSB*	8p22	*0.76*	*0.84*	*0.79*	*0.85*	0.94
129	*GATA3*	10p15	*0.76*	*0.56*	*0.79*	*0.79*	0.92
195	*CDH1*	16q22.1	*0.79*	*0.65*	*0.76*	*0.7*	*0.86*
200	*stSG30213*	16q tel	*0.66*	**1.29**	*0.82*	*0.79*	*0.82*
203	*WI-14673*	17p tel	*0.78*	*0.84*	*0.85*	*0.81*	*0.83*
229	**LAMA3**	18q11.2	**1.56**	1.02	**1.24**	**1.2**	**1.31**
242	*GLTSCR2,SULT2A1*	19q13.32	*084*	*0.77*	0.99	*0.85*	*0.84*

Bold, gain; italic, loss.

detected by metaphase CGH. In all 8 MPNSTs, the total aberration number was 138 with a mean value of 17.3 (range, 9-24) aberrations per sample. The number of gains and losses were 110 (mean, 13.8) and 28 (mean, 3.5), respectively. The altered chromosomal regions in MPNSTs are summarized in Fig. 1. The most frequent gains were located in 4q26, 6q (7 tumors each), 4q12-q26, 11q14, 15q21, 21q21 (6 tumors each), 5p14, 5q21, 11q14-q22, 12q21, and 14q21 (5 tumors each). The most frequent losses were seen in 16q21 (3 tumors), 1p36, 1q41-qter, 10p12-pter, 11q23, 16p13-pter, 17p13, and 19p13 (2 tumors each). There were no differences regarding the frequency and distribution of chromosomal aberrations between the 3 sporadic and 5 NF1-associated MPNSTs. The youngest patient (case 7) with a rush tumor progression had the highest number of aberrations among the 8 patients.

In contrast, metaphase CGH detected genomic abnormalities in 5 of 8 schwannomas (63%). The total aberration number was 17 (average, 2.1 per sample). The number of losses (15) was larger than that of gains (2). The most common alteration was loss of a whole arm of 22q, which was found in 5 of 8 (63%) schwannomas. Loss of 8p and loss of 9p was observed in 2 cases, each. The altered chromosomal regions in schwannomas are summarized in Fig. 2.

Microarray CGH. In order to investigate these chromosomal aberrations with a higher resolution and sensitivity, microarray CGH was employed to identify the genes with copy number aberration in a series of 5 MPNSTs. These 5 cases were selected among 8 MPNSTs with abundant aberrations. Aberrations were detected in all 5 MPNSTs. Table III demonstrates the results of microarray CGH and a summary of the results. Genes detected as a gain in ≥4 cases included EGFR, DAB2, MSH2, 2PTEL27, DDX15, CDK6, and LAMA3; genes identified as a loss in ≥4 cases included CDH1, GLTSCR2, EGR1, CTSB, GATA3, stSG30213, and WI-14673.

To calculate a concordance rate of metaphase CGH and microarray CGH, we compared the locus which was altered in microarray CGH with the result of metaphase CGH. The average concordance rate was 66% (individual cases: 58, 76, 55, 86, 57%).

Discussion

Five of 8 schwannomas had a loss in the long arm of chromosome 22. The loss of 22q, which harbors NF2, has been the only common genetic alteration reported in schwannomas to date (10-15). The NF2 gene may play an important role in the pathogenesis of schwannomas without involvement of other chromosomal regions (10). The loss of 22q12 was seen in schwannomas by metaphase CGH, but not frequently in MPNSTs (one case). The mechanisms of formation of schwannoma and MPNST seem to be quite different.

The aberration detected in MPNST by metaphase CGH has been reported in three series (16-19). In the current study, the number of chromosomal gains in MPNSTs detected by metaphase CGH was larger than that of losses. Our results were consistent with the data obtained by chromosomal banding analysis of MPNSTs (20-22). This tendency was also observed by previous studies (16,23). High incidence of gains suggests that the activation of proto-oncogenes may be predominant in MPNST during tumor progression as compared to inactivation of tumor suppressor genes. On the other hand,

Lothe *et al* (24) reported that the number of losses was larger than that of gains. This could be partially explained by the difference in the definition of the aberrations between the two studies.

Gains were frequently observed in the whole arm of chromosome 4 and a long arm of chromosome 6 (7 cases each). In these chromosomes, genes such as DDX15, PDGFRA, KIT, ESR and MYB are located. The gains in chromosomes 7 and 8 were less frequent; however, these gains mostly consisted of high-level gains. Although gain in the chromosome 17 which included p53 (17p13.1) was reported to be frequent (16,25), in the current study, it was observed in only 2 cases. Moreover, microarray CGH revealed a loss of p53 (17p13.1) in 3 cases. The gain of 8q was observed in 4 cases, including 3 high-level gains in the current study. According to Schmidt *et al* (25), and Mechtersheimer *et al* (16), the frequent gains included chromosomes 5p, 7, 8q, 15q, 12q, and 17q and losses included 9p, 13q, and 1p. In contrast, Koga *et al* reported a different tendency to that of previous reports; the number of losses was larger than that of gains. The losses in chromosomes 17, 19, and 22q were frequently seen in the report (18); however, in our analysis, they were seen in only one or two cases.

As for correlation between the clinical course and experimental results, the number of cases is too small to make a statistical analysis. However, the patient who was youngest and had rush clinical course had a large number of aberrations. The patient developed multiple pulmonary metastases and a local relapse in a short period after the tumor excision. Further analysis is demanded to clarify these relations in the future.

Aberrations of several genes were detected in 5 MPNSTs by microarray CGH. The genes detected as gains in all 5 cases were EGFR (epidermal growth factor receptor, 7p12) and DAB2 (disabled homolog 2, 5p13). EGFR expression being associated with the development of schwann cell-derived tumors has already been reported in a few papers (26,27). Although schwann cells usually lack EGFR, NF1-related tumors express EGFR, and the tumor progression was stimulated by EGF *in vitro*. Inhibition of the EGFR molecule (e.g. antagonists to the EGF receptor) could become a useful treatment for MPNSTs. Mitogen-responsive phosphoprotein, DAB2 (disabled homolog 2) located in 5p13, is expressed in normal ovarian epithelial cells but is down-regulated or absent from ovarian carcinoma cell lines (28). However, it has not been reported in neurogenic tumors. Gains of the following genes were detected in 4 of 5 MPNSTs: MSH2 (mismatch homolog 2), DDX15 [DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 15], CDK6 (cyclin-dependent kinase 6), and LAMA3 (laminin, alpha 3).

The genes detected as loss were as follows: CDH1 (cadherin type 1, 16q22.1); tumor supressor gene, EGR1 (early growth response 1, 5q31.1); CTSB (cathepsin B, 8p22); GATA3 (GATA binding protein 3, 10p15); and GLTSCR2 (glioma suppressor candidate region gene 2, 19q13.32). CDH1 mutates in gastric, breast, colorectal, thyroid, and ovarian cancers (29-31). Cadherin is the Ca^{2+}-dependent cell adhesion which has been known to be expressed in normal and reactive schwann cells. Loss of function of cadherin 1 is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis. Aberrations of EGR1, CTSB, Cathepsin D, GLTSCR2, and GATA3 have not been identified in MPNST-related papers, excluding GLTSCR2 aberration in glioma development (32).

Gain of 8q may be associated with gain of MYC (8q24.12-q24.13). The gain of 8q was seen in 4 of 8 cases by metaphase CGH in this study; however, microarray CGH did not reveal a gain of MYC in any of the 5 cases. MYB [v-myb myeloblastosis viral oncogene homolog (avian), 6q22-q23] aberration was also suspected in 7 cases by metaphase CGH in the current study, but it wasn't detected by microarray CGH. Prevalence of microarray CGH may lead to the more precise analysis of genomic analysis.

When comparing the results of metaphase and microarray CGH, the average concordance rate between the 2 techniques was 66% (range, 55-86%). This was mainly because losses could not be detected by metaphase CGH as much as by microarray CGH. If the gain and loss coexist in a narrow lesion, they can't be detected due to low resolutional activity of metaphase CGH. Hybridization to an array of mapped sequences instead of metaphase chromosomes could overcome the limitations of metaphase CGH. Metaphase CGH could be a reliable screening method before applying microarray CGH. Microarray CGH targets the genes with which we can prepare and construct the cDNA array. On the other hand, metaphase CGH could cover a whole DNA to detect the aberration where the regions contain unknown genes.

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