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Original Article

Scanning all chromosomal abnormalities with microarray-based comparative genomic hybridization in differential diagnosis of pediatric cancers

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Abstract: Objective: Despite conventional histopathological and immunohistochemical methods, difficulties may be experienced in the differential diagnosis of pediatric cancers, especially in small round-cell undifferentiated tumors. In these cases, the determination of chromosomal abnormalities may be helpful. The aim of this study was to evaluate the place of the whole genome array comparative genomic hybridization method in pediatric cancers where difficulty is experienced in differential diagnosis. Method: In Comparative Genomic Hybridization (CGH), 135,000 probes were scanned as 3 probes per gene in all genomes. It was possible to analyze paraffin block tissues obtained from the archive of the Pathology Laboratory of Dr. Behcet Uz Children's Hospital. DNA extraction was made from the paraffin blocks of 24 cases where difficulty had been experienced in making the differential diagnosis and in each case, comparisons with the control samples were made for all anomalies in all chromosomes using microarray technology. Results: Together with the typically observed chromosomal anomalies, additional derangements with debatable importance were determined. Conclusion: The whole genome CGH method may be useful in pediatric cancers where difficulties are experienced in making differential diagnoses. Since technical difficulties are experienced in the examination of paraffin-embedded tissue samples, storing fresh tissue samples from each tumor will be helpful for genetic and molecular examinations.

Keywords: Comparative genomic hybridization (CGH), pediatric cancers, small round cell malignant tumors, differential diagnosis

Introduction

Malignancies are one of the important causes of death in children. In developed countries, less than one percent of cancers occur in children under 15 years of age. The overall cancer prevalence is between 70-160 per million people under the age of 15 years [1]. In developing countries such as our country, malignancies are the fourth most common cause of death. Differential diagnosis is more important in childhood malignancies because of the longer life expectancy of children [1].

Primitive small round cell tumors observed in childhood belong to heterogeneous malignant tumor groups that constitute 0.1% of childhood malignant tumors. These tumors mainly seen in childhood may be listed as neuroblastoma, sarcomas, including rhabdomyosarcomas, non-rhabdomyosarcoma soft tissue sarcomas, Ewing sarcoma (ES)/primitive neuroectodermal tumor (PNET), desmoplastic small round cell tumor (DSRCT) and malignant rhabdoid/teratoid tumor (RT) [1-3].

The whole genome-comparative genomic hybridization technique is a cytogenetic method based on FISH demonstrating fluorescent color differences obtained by a linking test (patient), and reference DNA samples stained with different fluorescent dyes to normal chromosomes. With this method, structural chromosomal anomalies that cannot be detected by standard

techniques can be identified more reliably, allowing both the comparison of at least two genomes with each other and the analysis of the whole genome in a single experiment [4-6].

The aim of this study is to investigate the role of whole genome-comparative genomic hybridization in pediatric cancers posing difficulties in differential diagnosis.

Materials and methods

In the Pathology Laboratory of Dr. Behçet Uz. Children's Hospital, DNA samples were extracted from the paraffin blocks of 24 patients where difficulty was experienced in the differential diagnosis, and a CGH analysis was performed. The anticipated chromosomal anomalies were compared with the clinicopathological findings.

In the comparative genomic hybridization analysis, the differences in the number of copies in the whole genome or in a region on the genome with a referenced genome were determined using NimbleGen CGH arrays. The DNA of 24 patients (test DNA) and normal DNA (control DNA) to be analyzed for CGH were labeled with different fluorochromes and hybridized with metaphase chromosomes obtained from normal cells. Differences in fluorescence intensities across chromosomes in the reference metaphase domain refer to the differences in the number of copies (amplifications or deletions) in the tumor DNA. If DNAs showing different fluorescent phenotypes overlap with normal metaphase chromosomes, they are have a blue-orange color.

If there is a deletion in the test DNA, hybridization does not occur in this region, so this region appears red. In contrast, if there is an amplification in the DNA, the region is appears green and is brighter than the other regions because hybridization is more intense in this region. Chromosome hybridization profiles were calculated according to hybridization rates using a special image analysis software program.

Results

Our study population consisted of patients with Ewing sarcomas (n = 10), neuroblastomas (n = 5), and undifferentiated malignant tumors (n = 9) (**Table 1**). Previous diagnoses of RT (n = 2), DSRCT (n = 2), and RMS (n = 1) were considered in 5 cases with diagnoses of undifferentiated malignant tumors. The ages of the patients ranged from 2 months to 18 years. The mean age of the patients was 8.7 ± 5.3 years. The series consisted of 10 boys (41.7%) and 14 girls (58.3%). The tumors were localized in the kidneys and adrenal glands (n = 6), the abdominal cavity (n = 4), the chest wall (n = 4), the soft tissue of the hip or of an extremity (n = 3), the lungs or pleura (n = 2), the pelvic region (n = 2), and the liver (n = 1). The location of the tumor was unknown in 2 cases sent for the consultation (**Table 1**).

In all, 3 male and 7 female Ewing sarcoma patients were analyzed (**Figure 1**). The median age of the patients was 8. 6 years. The oldest and the youngest patients were 14 years and 1 year old, respectively. The tumors were located in different body parts. These tumors were localized in the extremities (n = 2), the lungs (n = 1), and in the soft tissue of the trunk (n = 7). The CGH analysis frequently detected 8 gene domains (commonly shared gene domains found in 6 and 7 cases with Ewing sarcoma) (**Table 2**).

In our series, 1 male, and 4 female patients with a median age of 7.2 years (range, 2-16 years) received the diagnosis of undifferentiated neuroblastoma. The tumors were localized in the adrenal glands in 3 cases. In the CGH analysis, 8 gene regions (commonly shared in 4 or 5 of 9 cases) were often detected (**Table 3**).

In addition, 9 cases were diagnosed as undifferentiated malignant tumors. Among the cases diagnosed as undifferentiated tumors, the previous diagnoses of RT (n = 2), DSCRT (n = 2), and RMS (n = 1) were considered. In the CGH analysis, 8 gene regions (commonly shared in 4 or 5 of 9 cases) were often detected (**Table 4**).

Discussion

Due to the heterogeneous structure of the primitive small round cell tumors, the limitation in the number of tissue specimens sampled in the biopsy, and the overlapping morphological and even immunohistochemical findings, the differential diagnosis may be challenging. Therefore, molecular analyses may be needed for the diagnosis of these tumors [7]. A single translocation or deletion analyzed with a single probe may not always yield precise results. Because the chromosomal anomaly in question may not be of a uniform type, variations

Table 1. Patient demographic data

CASE	CASE NUMBER	AGE (year)	SEX	LOCATION	DIAGNOSIS	SURVIVAL
1	1A1	4	F	Iliac bone region	Ewing sarcoma	Not known
2	1A2	4	M	Abdomen	Undifferentiated malignant tumor	Not known
3	1A3	11	M	Thoracic wall	Ewing sarcoma	Not known
4	1A4	9	M	Pelvic region	Undifferentiated malignant tumor	Not known
5	1A5	8	M	Sacral region	Ewing sarcoma	Died
6	1A6	0	F	Liver	Undifferentiated malignant tumor	Not known
7	1A7	18	F	Not known	Ewing sarcoma	Alive
8	1A8	17	F	Not known	Ewing sarcoma	Died
9	1A9	13	M	Lung/pleura	Undifferentiated malignant tumor	Died
10	1A10	2,5	F	Pelvic region	Undifferentiated malignant tumor	Not known
11	1A11	10	F	Kidney	Undifferentiated malignant tumor	Not known
12	1A12	7	F	Thoracic wall	Ewing sarcoma	Not known
13	2A1	13	F	Abdomen	Ewing sarcoma	Not known
14	2A2	3	M	Kidney	Undifferentiated malignant tumor	Died
15	2A3	3	M	Kidney	Undifferentiated malignant tumor	Died
16	2A4	15	M	Shoulder	Ewing sarcoma	Alive
17	2A5	15	F	Lung/pleura	Ewing sarcoma	Died
18	2A6	14	F	Thoracic wall	Ewing sarcoma	Alive
19	2A7	5	M	Abdomen	Undifferentiated malignant tumor	Alive
20	2A8	7	F	Adrenal gland	Neuroblastoma	Alive
21	2A9	16	F	Abdomen	Neuroblastoma	Not known
22	2A10	2	M	Adrenal gland	Neuroblastoma	Alive
23	2A11	7	F	Adrenal gland	Neuroblastoma	Alive
24	2A12	5	F	Thoracic wall	Neuroblastoma	Alive

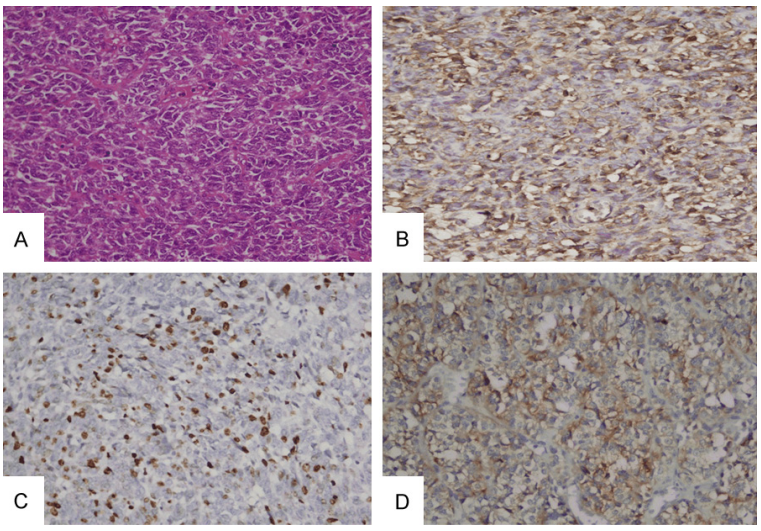

Figure 1. (A) General appearence of ES/PNET tumor cells (HE × 200). Immunohistochemical stains in the same tumor: (B) Ki67 proliferation index, (C) Expression of CD99 (DAB × 200), and (D) Expression of Synaptophysin (DAB × 200).

can be found, or, it may be necessary to look at multiple sites for the differential diagnosis. In these cases, CGH may help detect chromosomal anomalies [6].

In our ES/PNET patient cohort, the number of copies were most often altered in genes srGAP2, RGPD5, NPHP1, GTF-2IRD2, PDXDC1. srGAP2 is a member of the SLIT-ROBO Rho GTPase activating protein family, which regulates cell differentiation and neuronal migration. The gene is also located in region 1q. The deletions in 1q regions were also observed in previously performed CGH analyses [8]. RGDP5 is a nuclear membrane-associated protein that regulates cellular functions through interactions with other proteins. NPHP1 interacts with Crk-associated substrates and controls cell division, cell-cell, and cell-matrix

Table 2. Copy number altered genes among patients with ES/PNET

SYMBOL	GENE NAME	LOCATION	1A1	1A3	1A5	1A7	1A8	1A12	2A1	2A4	2A5	2A6	Total
srGAP2	SLIT-ROBO Rho GTPase-activating protein 2	1q32.1	del	del	del	del	del	x	del	del	x	x	7/10
RGPD5	RANBP2-like and GRIP domain containing 5	2q13	del	del	x		del	del	del	del	del	x	7/10
NPHP1	nephrocystin 1	2q13	del	x	x	del	del	del	del	del	del	x	7/10
GTF2IRD2	GTF2I repeat domain containing 2	7q11.23	del	del	x	del	x	del	del	del	amp	x	7/10
PDXDC1	pyridoxal dependent decarboxylase domain containing 1	16p13.11	del	del	x	del	del	del	x	del	del	x	7/10
MALL	T cell differentiation protein like	2q13	del	del	del	del	del	x	del	x	x	x	6/10
ANKRD30A	Ankyrin repeat domain 30A	10p11.21	del	del	x	del	x	del	x	del	del	x	6/10
PTPN20	PTPN20 protein tyrosine phosphatase, non-receptor type 20	10q11.21	del	del	del	x	del	x	x	del	del	x	6/10
USP22	ubiquitin specific peptidase 22	17p11.2	x	del	x	del	x	del	del	del	del	x	6/10

Table 3. Copy number changes among patients with neuroblastoma

SYMBOL	GENE NAME	LOCATION	2A8	2A9	2A10	2A11	2A12	Total
DEFB107A	Defensin beta 107A	8p23.1	del	del	del	del	del	5/5
MAGEA12	MAGE family member A12	Xq28	del	del	x	del	del	4/5
SPANXD	SPANX family member D	Xq27.2	del	x	del	del	del	4/5
SAGE1	sarcoma antigen 1	Xq26.3	del	del	del	del	x	4/5
DEFB106A	Defensin beta 106A	8p23.1	x	del	del	del	del	4/5
DEFB103B	Defensin beta 103B	8p23.1	del	del	del	x	del	4/5
RGPD5	RANBP2 like and GRIP domain containing 5	2q13	del	del	x	del	del	4/5

adhesion signaling. GTF2IRD2 is a transcriptional factor under the control of the retinoblastoma protein.

MALL participates in raft-mediated trafficking in endothelial cells. MAL1 interacts with caveolin and is localized in glycolipid or cholesterol-enriched membrane rafts. PTPN20 is a member of the protein tyrosine phosphatases and is involved in actin polymerization. USP22 is an interesting gene that regulates various cellular and organismal processes such as cell proliferation, apoptosis, and malignant transformation. It also deubiquitinates histones H2A and H2B, thereby acting as a coactivator. USP22 participates in the activation of several oncogenes such as MYC and contributes to stemness in colorectal carcinogenesis as well as the epithelial mesenchymal transition in osteosarcoma cell lines. Altered levels of ANKRD30A expressed in epithelial cells and in the testes have been found to be associated with breast cancer. TPTE is a PTEN-related tyrosine phosphatase and plays a role in the endocrine signal transduction pathways and in the spermatogenic function of the testes. CTSE encodes peptidase involved in antigen processing and the maturation of secretory proteins [1, 8].

Even with the availability of advanced molecular and immunohistochemical diagnostic methods, the diagnosis of poorly differentiated and undifferentiated neuroblastomas may be especially difficult. The defensin beta 107A gene, where copy number variations were most frequently detected in the CGH analysis performed in our patient series, and the defensin family produced by neutrophils, are antimicrobial, and cytotoxic peptides are effective on the immune system. There is evidence that abnormal defensin expression is related to a susceptibility to infectious diseases and inflammatory disorders. The loss/deletion of defensin beta 107A has been especially observed in breast, cervix, colon, and bladder cancers [10-12].

The MAGE family member A12 gene is closely related to several other genes clustered on the X chromosome. It has been reported that these genes may be overexpressed in melanoma and breast cancer tumors [13, 14]. However, in our study, a deletion was detected in the MAGE family member A12 gene in neuroblastoma cases. The SPANX family member D sarcoma antigen 1 plays a role in the formation of mature spermatozoa. It is necessary to initiate the sequence of molecular and morphological changes in the germ cells. This gene is a member of the gene family associated with the SPANX cancer/testis localized in a cluster on the X chromosome. SPANX genes encode diversely differentiated testis-specific proteins that are localized in different subcellular compartments. Although the specific function of this gene has not yet been determined, polymorphisms in this gene may be associated with a susceptibility to prostate and breast cancer [15, 16].

Defensin beta 106A is an interesting gene that regulates the processes of stimulating the immune system, especially in the acquired immune response [17]. Defensin beta 103B is also effective in the immune system. A beta-defensin 3, expressed in the epithelium in response to various stimulations, including human papilloma virus infection, has recently been found to be overexpressed in head and neck cancers and cervical cancers and has been shown to exhibit tumorigenic activities [17, 18]. However, the RGPD5 gene is a small GTP binding protein of the RAS superfamily, thought to control various cellular functions associated with nuclear membranes and interactions with other proteins. Its relationship with cancerogenesis has not been detected yet [19].

Table 4. Copy number changes among patients with undifferentiated malignant tumors

SYMBOL	GENE NAME	LOCATION	1A2	1A4	1A6	1A9	1A10	1A11	2A2	2A3	2A7	Total
ANKRD30B	ankyrin repeat domain 30B	18p11.21	del	X	X	del	del	del	del	x	x	5/9
SLC6A4	solute carrier family 6 member 4	17q11.2	del	del	x	x	del	del	del	x	x	5/9
PPIB	peptidylprolyl isomerase B	15q22.31	del	del	x	del	x	del	del	x	x	5/9
HERC2	HECT and RLD domain containing E3 ubiquitin protein ligase 2	15q13.1	del	del	x	del	del	del	x	x	x	5/9
TDO2	tryptophan 2,3-dioxygenase	4q32.1	amp	amp	x	del	del	del	x	x	x	5/9
IGSF21	immunoglobin superfamily member 21	1p36.13	x	x	x	del	del	x	del	del	x	4/9
CAMK2B	calcium/calmodulin dependent protein kinase II beta	7p13	x	x	x	del	del	x	del	del	x	4/9
POM121	POM121 transmembrane nucleoporin	7q11.23	del	x	x	del	del	del	x	x	x	4/9

Difficulties encountered in making the differential diagnoses of undifferentiated malignant tumors, especially in the group of small round cell malignant tumors should be expected. Even immunohistochemical and molecular tests may not show the cases' degrees of differentiation. In such cases, the CGH analysis can be used both to detect DNA copy number variations and to make a differential diagnosis. In 9 cases of our patient series, we could not arrive at a definite differential diagnosis. The immunoglobulin superfamily member 21 gene, which is one of the genes most frequently demonstrating copy number variations in the CGH analysis, encodes a protein that is a member of the immunoglobulin superfamily. These proteins are usually found in cell membranes and act as receptors in immune response pathways. Its association with carcinogenesis has not been determined yet [20].

The product of the calcium/calmodulin-dependent protein kinase II beta (CAMK2B) gene belongs to the serine/threonine protein kinase family and the Ca (2+)/calmodulin-dependent protein kinase subfamily. Calcium signaling is very important for many different aspects of plasticity in glutamatergic synapse. In one study, the dysfunction of CAMK2B was associated with osteoblastic bone metastases of the prostate, and it is closely related to osteoblastogenesis of human mesenchymal stem cells [21]. POM121 transmembrane nucleoporin is a component of the nuclear pore complex (NPC). The NPC functions in the regulation of nucleus-cytoplasmic transport, transcription, and the stability of the genome. However, their functional roles in cancer have been poorly understood. There is evidence in the literature that NPC may play a role in the development of primary metastatic prostate cancer [22].

Ankyrin repeat domain 30B gene expression is contained in the testes. The solute carrier family 6 member gene expresses the integral membrane protein that transports serotonin from synaptic domains to presynaptic neurons. It encodes a transport protein that mediates the uptake of long-chain fatty acids in the cell. The function of these proteins is mainly related to lipid biosynthesis, the fatty acid metabolic process, and fatty acid transport. It has been shown to play a role in depression sensitivity in people with aggressive behavior and emotional trauma, especially in Alzheimer's patients. In addition, the deletion of this gene may contribute to the development of breast and thyroid cancer and may be effective in reducing survival in patients with colorectal cancer [23-25]. Peptidylprolyl isomerase B, a gene product cyclophilin B (CypB), is an endoplasmic reticulum protein that binds to cyclophilin A, which is a member of the cyclophilin family [26, 27]. CypB has peptidyl-prolyl cis-trans isomerase (PPIase) activity. PPIases catalyze the cis-trans isomerization of proline and peptide bonds in oligopeptides and accelerate the folding of proteins. The encoded protein is a cyclophilin A (CypA) binding protein and may play a role in cyclophilin mediated immunosuppression. CypA, the protein produced by this gene, has been reported to be upregulated in breast, stomach, cervical, and pancreatic cancers [28-31]. In particular, it has been reported to regulate the growth and survival of gastric cancer cells and may be related to the recovery of tumor growth and anti-apoptotic functions, and it has been noted as a very important link in the pathogenesis of cancer [26]. In addition, the overexpression of CypB inhibits the activation of antioxidant enzymes in the cell and the resulting reduction of oxidative stress, especially the neurogenic cytotoxicity by mitogen-activated protein kinase through the c-Jun N-terminal kinase pathway [27].

The RLD domain-containing E3 ubiquitin protein ligase 2 gene (HERC2) belongs to the HERC gene family, which encodes a very large group of proteins containing a large number of structural domains. The mutation of the HERC2 gene leads to severe neurodevelopmental disorders. Furthermore, in non-small cell lung cancer, HERC2 facilitates the incorporation of the RNF8-UBC13 complex so as to introduce BRCA1 to DNA damage sites. Moreover, its association with cancer development has been reported [32]. The tryptophan 2.3-dioxygenase (TDO2) gene encodes a heme enzyme that plays a critical role in tryptophan metabolism. The increased activity of the encoded protein and the subsequent production of kynurenine may also play a role in cancer by suppressing antitumor immune responses. In one study, the overexpression of TDO2 was observed in the cancer stem cells of esophageal squamous cell carcinoma, and its probable association with poor prognosis was noted [33, 34].

In conclusion, the CGH array may be a useful method for the detection of DNA copy number alterations among pediatric patients with undifferentiated malignant tumors. In our ES/PNET dataset, the copy number alterations were mostly found in genes related to cytoskeletal elements, migration, and protein trafficking in our cohort.

Changes in the number of copies in our neuroblastoma series were mostly found in genes that regulate the stimulation of the immune system [11, 12, 17, 18]. Changes in the number of copies of undifferentiated malignant tumors were found in genes that may be important in mesenchymal stem cell differentiation and in genes that may have played a role in tumor development through the suppression of immune responses and that may cause oxidative stress [21, 26-31]. In order to better understand the role of these genes in tumorigenesis and their contribution to the differential diagnosis, it is necessary to examine the functions of genes in larger series.

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Disclosure of conflict of interest

None.

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