3. INTERNATIONAL BAKU SCIENTIFIC RESEARCH CONGRESS

October 15-16, 2021/ Baku, Azerbaijan,
BAKU EURASIA UNIVERSITY

THE BOOK OF FULL TEXTS

EDITOR
Prof. Dr. Gulzar İBRAHİMOVA

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MOLECULAR HETEROGENEITY IN NEUROBLASTOMA AND CLINICAL SIGNIFICANCE (ON BEHALF OF THE TURKISH PEDIATRIC ONCOLOGY GROUP)

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ABSTRACT:

Aim: Heterogeneity in cancer means that sections of the tumor display different behavior. Neuroblastoma is a disease where heterogeneity causes problems with treatment. This study aims to find about relation of neuroblastoma molecular heterogeneity with clinical properties.

Methods: Fresh tumor tissue or formalin fixed paraffin embedded samples were selected from tissues that were sent to our department for molecular investigation according to the TPOG 2009 NB protocol. The metachronous group comprised all patients with samples taken from the same patient at different times at least one week apart. The synchronous group comprised randomized patients with sufficient amount of tissue for double sampling in samples taken from surgical material. Molecular investigations were performed prospectively at time of diagnosis in neuroblastoma cases in tumor tissue in two different areas or tissues (synchronous) or at two different times (metachronous). Heterogeneity was questioned with investigation in different samples of MYCN amplification, 1pLOH, 11q deletion and 17q gain identified with real-time PCR and DNA ploidy identified with flow cytometry.

Results: Heterogeneity was observed in 28.6% of cases for MycN, 45.5% for 1pLOH, 23.5% for 11qdel, 47.2% for 17q gain and 33% in DNA ploidy in metachronous group and %20 of cases for MycN, 44.4% for 1pLOH, 44.8% for 11qdel, 42.3% for 17q gain and 50% in DNA ploidy in synchronous group. There were cases where examinations from different tissues might have changed the risk classification.

Conclusion: Our findings support the view that it will be beneficial to perform molecular investigation by sampling from the tumor as much as possible in situations like lack of full response at the end of recurrence treatment or in different clinical periods in neuroblastoma.

Key words: Neuroblastoma, MycN, molecular heterogeneity

INTRODUCTION:

Heterogeneity in cancer means that sections of the tumor display different behavior as a result of tumor cells having different characteristics(1-3). Metachronous heterogeneity means samples taken from the same patient at different times display different biological features. The simplest example of this is the disappearance of treatment response of a tumor due to survival of resistant cells after chemotherapy. Synchronous heterogeneity means that samples taken from different regions of the same tumor display different biological features(4) . The use of medications targeting specific molecules to reduce side effects in current medical approaches has gained importance with the identification and interpretation of heterogeneity(5). The main mechanism in resistance against molecular medications is considered to be biological heterogeneity (6, 7). As a result, identification of different clones in targeted treatment and planning for these clones has gained importance in molecular treatment.

Neuroblastoma shows pronounced heterogeneity(8) and is a disease where this heterogeneity causes problems with treatment (9). Contrary to good survival in the low- and moderate-risk groups, treatment efficacy is limited in the high-risk group. In Turkey, the general survival rates for neuroblastoma are 100%, 75.8%, 34.1%, 6.5% and 59.4% for stage 1, 2, 3, 4 and 4S, respectively (10, 11). When Turkish Pediatric Oncology Group (TPOG) neuroblastoma data from 2009 are assessed, the 3-year overall survival (OS) in the low-risk group was 98%, and event-free survival (EFS) was 90%. In the moderate-risk good histology group, 3-year OS is 100% while EFS was 93%. In the moderate-risk poor histology group, 3-year OS was 90% with EFS 76%. In the high-risk group, conventional CT branch had 3-year

OS of 53% and EFS of 37%, while the high-dose CT branch had OS of 59% and EFS of 33% (12). Though neuroblastoma contains a variety of molecular targets like ALK and MYCN (13, 14), efficacy of molecular treatment due to pronounced heterogeneity is a topic of current research (8, 15-17).

Studies related to molecular heterogeneity are based on investigation of pathologic sections and determination of cell features. As chromosomal aberrations are frequently observed in neuroblastoma, FISH and qPCR methods are common in heterogeneity studies. Currently, guidelines recommend performing two molecular studies in at least two areas if neuroblastoma has macroscopic areas with different colors (18). Though the presence of MYCN with molecular heterogeneity is known for a long time, how this heterogeneity should be interpreted clinically still has not been clarified as necessary (16).

In our study, molecular investigations were performed prospectively at time of diagnosis in neuroblastoma cases in tumor tissue from two different areas or tumor tissues (synchronous) or at two different times (metachronous). Heterogeneity was questioned with investigation in different samples of MYCN amplification, 1pLOH, 11q deletion and 17q gain identified with real-time PCR and DNA ploidy identified with flow cytometry. The correlation between presence of heterogeneity with clinical findings was investigated with the aim of investigating the effect of molecular heterogeneity in neuroblastoma on clinical risk classification.

MATERIALS AND METHODS:

Patient and samples

Our research received ethics committee permission and patient relatives signed voluntary consent forms.

The universe comprised 1300 fresh tumor tissue or formalin fixed paraffin embedded samples sent to our department for molecular investigation according to the TPOG 2009 NB protocol (19) from 2012-2020. Two samples taken from the same patient who had molecular investigation were included in this study in two groups. The metachronous group comprised all patients with samples taken from the same patient at different times at least one week apart. The synchronous group comprised randomized patients with sufficient amount of tissue for double sampling in samples taken from surgical material. Samples were chosen from differently colored macroscopic areas with microscopic imaging. For the synchronous group,

the first sample was taken from less differentiated dark-color areas (A sample), while the second sample was taken from well-differentiated light-color areas (B sample). The clinical data for 73 patients included in the study (37 double sample metachronous group, 34 double sample synchronous group) were recorded from the TPOG archive and patient information.

Molecular Investigations

DNA isolation

DNA isolation was performed using kit (Roche High Pure PCR Preparation Kit). Fresh tumor tissues were imprinted on a slide, stained with toluidine blue and investigated with a light microscope for tumor evaluation. Per sample, 3 mm diameter tissue was used. After mechanical disintegration of tissues, they were left in proteinase K and lysis buffer for 2 hours at 55 C. The DNA samples isolated with the spin column method according to manufacturer's instructions quality were assessed with Qubit in terms of quality.

Real Time PCR

DNA obtained from tumor tissues were assessed in terms of MYCN amplification (2p24), 1p36 LOH, 11q23 deletion and 17q25 gain with real-time PCR reactions. Identification of gains or losses in these regions were assessed using primer and enzyme mixtures containing labeled probes designed specific to these regions. For MCYN real-time PCR, the enzyme mixture containing 5'GTGCTCTCCAATTCTCGCCT-3' and 5'-GATGGCCTAGAGGAGGGCT-3' primer pair and the '5'FAM-CACTAAAGTTCCTTCCACCCTCTCCT-TAMRA-3' Taqman probe designed specific to the MYCN gene in the 2p24.3 region was used. To assess MYCN amplification, the NAGK (n-acetylglucosamine kinase) gene found in the 2p13.3 region of the same chromosome was taken as reference. For the NAGK gene, the enzyme mixture containing 5'-TGGGCAGACACATCGTAGCA-3' and 5'-CACCTTCACTCCCACCTCAAC-3' primer pair and '5'-VIC-TGTTGCCCGAGATTGACCCGGT-TAMRA-3' ' Taqman probe was used. For the GNB1 (guanine nucleotide binding protein (G protein)) gene found in the 1p36.33 region, the enzyme mixture with 5'-AGCCAGTGGCAAATGCATT-3' and 5'TCTCTGCAGCCCTACCATTGA-3' primer pair and '5'-FAMAGCAAATCAAGACATCATGTAAACGCTCA-BBQ(tamra)' TaqMan probe designed for this gene was used. For losses in this region, the NGFB (nerve growth factor (beta polypeptide)) gene found in the 1p13.1 region of the same chromosome was taken as reference. For the NGFB gene, the enzyme mixture containing 5'-

TTCTATCCTGGCCACACTGAG-3’ and 5’- TTCTGACTTGCCCTCTCAGGT-3’ primer pair with 5’-FAM(VIC)- TTG CCA AGG TCC TCC CTC TGC AGC T-BBQ(tamra) Taqman probe was used. For 11q23 deletion analysis, the enzyme mixture designed specific to the ARCN1 (archain 1) gene found in the 11a23.3 region containing 5’-ATCTGGAGGCAGCACAGCT -3’ and 5’- TACACTGGATTATACCTGGCTGG-3’ primer pair with ‘5’-FAM- CCATGATCACAGAGACCATCATTGAAA-BBQ(tamra)’ Taqman probe was used. For deletion in this region, the MYBPC3 (myosin binding protein C cardiac) gene found in the 11p11.2 region of the same chromosome was taken as reference. For the MYBPC3 gene, the enzyme mixture containing 5’-TGGTGTACGAGATGCGCGTC-3’ and 5’TCACCGATAGGCATGAAGGG-3’ primer pair with ‘5’-FAM(VIC)-TCAACGCCATCGGCATGTCCAGG-BBQ(tamra)’ Taqman probe was used. For 17q gain analysis, the enzyme mixture designed for the surviving gene found in the 17q25 region containing 5'-GGGCTGCCACGTCCAC-3' and 5'-GTCGTCATCTGGCTCCCA-3' primer pair with ‘5'FAM-TTCATCCACTGCCCCACTGAGAACGA-TAMRA-3' ‘ Taqman probe was used. Gains in this region were assessed by taking the TP53 gene located in the 17p13.1 region of the same chromosome as reference. For the TP53 gene, the enzyme mixture containing 5’TGTCCTTCCTGGAGCGATCT-3’ and 5’-CAAACCCCTGGTTTAGCACTTC -3’ primers with ‘5’-(FAM) Yakima yellow-CAGCCCCCGGCTCCGCTAGA-TAMRA-3’ ‘ Taqman probe was used. Control DNA was used normal DNA not containing these aberrations(custom standart DNA). Results were calculated as target/control proportional values and deltaCTs with presence or absence of aberrations assessed according to cut-off values. For MYCN amplification, 10 times positive amplification was assessed. For 1p36 LOH and 11q23 deletion <0.5 value was accepted as positive, while for 17q25 gain >1.3 value was taken as cut-off and results were recorded as positive or negative.

The transfer solution (containing tumor cells after tissue was removed) was centrifuged at 2000 rpm for 7 minutes, and tissue medium cells were obtained and suspended in freeze media (95% RPMI complete (88% RPMI+10% FBS+1% L-Glutamine+1% penicillin- streptomycin) +5% DMSO) for assessment of DNA index. These cells were stored at -20 ℃ until DNA index analysis. DNA index was identified with BD accuri flow cytometry using cell cycle kit. Flow cytometry analyzes light radiated at 564 and 606 nm by cells with routine stained by propidium iodide. The fluorescent histograms obtained are assessed for the presence of DNA aneuploidy using normal cells from peripheral blood mononuclear cells (PBMN). DNA index is obtained by dividing the relative DNA content mode in the abnormal

G0/G1 population by the relative DNA content in the normal G0/G1 population. Additionally, variation coefficients are given for each G0/G1 peak.

Findings were analyzed using SPSS 22.0. After descriptive statistics, cases were assessed for presence of heterogeneity with chi-square or Fisher exact test according to smallest groups and two-element parameters.

RESULTS:

In total, 71 double samples were obtained in this study from 71 cases. The metachronous group comprised 37 cases, while the synchronous group comprised 31 cases. The cases comprised 35 girls and 36 boys. The metachronous group comprised 3 low-risk cases, 6 moderate-risk cases and 15 high-risk cases, while the synchronous group comprised 10 low-risk cases, 2 moderate-risk cases and 8 high-risk cases. The clinical and molecular features of cases are summarized in Table 1.

Table 1: Distribution of metachronous and synchronous cases according to molecular heterogeneity percentages

	MYCN Heterogeneity	1pLOH Heterogeneity	11Q DEL Heterogeneity	17Q GAIN Heterogeneity	DNA PLOIDY Heterogeneity
METACHRON	28.6% (10/35)	45.5% (15/33)	23.5% (8/34)	47.2% (17/36)	33% (3/9)
SYNCHRON	20% (6/30)	44.4% (12/27)	44.8% (13/29)	42.3% (11/26)	50% (1/2)

In cases, 24.6% had MYCN heterogeneity, 45.0% had 1pLOH heterogeneity, 33.3% had 11qdel heterogeneity and 45.2% had 17qGain heterogeneity. DNA ploidy was heterogeneous in 36.4% of cases. Of patients with clinical data, 58.5% (33/51) were metastatic and 32.7% (16/49) were relapse. For patients with survival information available, 67.3% (33/49) had disease-free survival, 8.5% had disease survival and 22.4% died. In total, 6 cases had risk difference between samples. Two of these 6 cases were diagnosed with Stage 4S at time of diagnosis, which was later corrected to stage 4. One case was refractory so moved from the moderate-risk poor histology group to high risk. Three cases had variations in staging due to heterogeneity. All these cases were negative for MYCN with staging variations due to heterogeneity observed in terms of 1pLOH, 11qdel and 17qGain. All cases with MYCN heterogeneity were in the high-risk group (Table 2).

Table 2: Clinical Properties of the cases

		Mean age Month	Sex M:n F: n	MycN amp	1p LOH	11q del	17q gain	Heterogeneity
Synchronous	Low risk n:	15.22	M:3 F:6	0/9 cases	4/9 cases	1/9 cases	4/9 cases	1/9 for MycN, 4/9 for 1p LOH, 4/9 for 11q Del, 3/8 for 17q Gain, 1/1 for DNA ploidy
	Intermediate risk n:	45.83	M:5 F:1	0/6 cases	3/6 cases	3/6 cases	2/5 cases	3/6 for MycN, 2/5 for 1p LOH, 1/3 for 11q Del, 1/2 for 17q Gain
	High risk n:	48.91	M:7 F:5	5/12 cases	3/11 cases	5/12 cases	8/12 cases	5/11 for MycN, 3/9 for 1p LOH, 6/11 for 11q Del, 5/11 for 17q Gain, 0/1 for DNA ploidy
Metachronous	Low risk n:	29.75	M:3 F:1	0/4 cases	0/4 cases	1/4 cases	2/4 cases	1/4 for 1pLOH 1/4 for 17q gain
	Intermediate risk n:	13.80	M:0 F:5	0/5 cases	3/5 cases	1/5 cases	2/5 cases	3/5 for 1p LOH, 1/5 for 11q Del, 2/5 for 17q Gain,
	High risk n:	45.66	M:14 F:7	5/21 cases	9/20 cases	5/21 cases	8/21 cases	8/21 for MycN, 9/20 for 1p LOH, 5/20 for 11q Del, 10/21 for 17q Gain, 3/5 for DNA ploidy

In our study, the case coded 18 had a unique situation, and so it is appropriate to present findings independently. The female patient aged 12 months and 1 week was assessed in the low-risk group due to being stage 2B at time of diagnosis but was later included in the refractory treatment group due to lack of response to treatment. The patient died at 30 months and 2 weeks of age (survival duration 18 months 1 week). Two samples were assessed with Shimada poor pathology. Samples sent to us were tru-cut (NB407) and 3-days later resection material (NB413). Samples were assessed in the synchronous group (this case played the main role in determining the week limit), with no differences found in NB407 and NB413 (MYCN: Negative (<5), 1pLOH: Positive, 11qdel: Negative, 17qGain: Negative). Later, a second sample was taken from NB413 for better compatibility with the synchronous group (NB413-B). This sample found MYCN positive (139 times), 11qdel negative, 17qGain negative and 1pLOH could not be studied. This patient was assessed in the low-risk group

according to TPOG-2009 NB protocol (19); however, the MYCN elevation in the third sample from the patient leads to consideration of different genetic structuring in this region.

The chi-square test showed no correlation between gender and heterogeneity, risk differences and metastasis frequency. There was no correlation shown for metachronous-synchronous groups and heterogeneity, risk differences and metastasis frequency with the chi-square test. Though in cases with risk difference there were no cases without MYCN heterogeneity, the Fisher Exact test found p=0.190.

DISCUSSION:

In this study, molecular investigation was performed in tumor tissue taken from two different areas or tissues (synchronous) prospectively at time of diagnosis or two different times to investigate heterogeneity in different samples. Investigating the correlation between presence of heterogeneity and clinical findings was used to examine the effect of molecular heterogeneity in neuroblastoma on clinical risk classification and notable heterogeneity was identified. Our study used PCR for molecular investigation instead of the FISH method used in the literature and heterogeneity was compared with RT-PCR findings. Though MYCN heterogeneous cases are known in neuroblastoma(17, 20, 21), studies about other genetic markers are limited. In our study, 1pLOH, 11qdel and 17qGain heterogeneity was observed with frequency above 40% and this heterogeneity may affect clinical decision-making.

In the literature, the percentage of neuroblastoma patients with hetMNA identified with the FISH method is above 40% (22). In our study, we used PCR method for heterogeneity identification instead of FISH. As a result, we think lower heterogeneity rates were observed.

In our study, the aim was to assess heterogeneity in neuroblastoma in terms of time. It was considered that this may be helpful in creating a different approach to cancer heterogeneity. Due to intratumoral sample selection in the synchronous group, it has pioneering features for assessment of hetMNC neuroblastoma(21) in Turkey. The main cause of samples sent from clinics being recollected and resent is sample insufficiency, which is the main limitation due to severe deficient data for dependent group assessment of patients included in our study.

Investigation of specific genes in chromosomal regions monitored with more specific genetic markers for heterogeneity in neuroblastoma may ensure more effective research about the effect of heterogeneity in neuroblastoma. In this way, an effective increase may be provided in clinical prediction and treatment planning for high-risk neuroblastoma cases.

In conclusion, we think it will be beneficial to perform molecular studies more than once on several tissues in neuroblastoma cases. Our findings support the view that it will be beneficial to perform molecular investigation by sampling from the tumor as much as possible in situations like lack of full response at the end of recurrence treatment or in different clinical periods in neuroblastoma(23-25). As studies of fluid biopsy or bone marrow aspiration increase, more clarity will be gained about whether these can take the place of tumor tissue.

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