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Original Article

Molecular Heterogeneity in Neuroblastoma and Its Clinical Significance

Nöroblastomda Moleküler Heterojenite ve Klinik Önemi

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ABSTRACT

Objective: Tumor heterogeneity describes the differences between cancer cells in the same tumor sample. Neuroblastoma (NB) is a type of cancer where tumor heterogeneity complicates its treatment. This study aims to explore the role of molecular heterogeneity detected by routine molecular tests in NB.

Method: Seventy-one patients were included in the study. NB samples were chosen among 1,300 NB samples that were evaluated using molecular tests between 2012-2020 according to the guidelines of Turkish Pediatric Oncology Group Protocol. Molecular investigations were performed (total 142 samples) obtained from two different areas of the tumor (synchronous) or at two different times (metachronous). Heterogeneity was questioned for five tests: MYCN amplification, 1p36LOH, 11q23 deletion and 17q25 gain (identified with real-time polymerase chain reaction) and DNA ploidy (identified with flow cytometry).

Results: Heterogeneity was observed for MYCN in 22.53%, for 1p36LOH in 36.62%, for 11q23del in 29.58%, and for 17q25 gain in 40.85% of cases, while DNA ploidy was heterogeneous in 36.4% of cases. Molecular heterogeneity did not show statistical difference among metachronous and synchronous cases. High-risk cases more frequently displayed molecular heterogeneity without any statistically significant difference between both groups.

Conclusions: Our findings support the fact that molecular heterogeneity either exists in different areas of a tumor or seen in the same tumor at different times. It will be beneficial to perform more than one molecular analysis on the tumor tissue specimens. In addition, recurrences or re-biopsy specimens from metachronous metastases shall be re-evaluated using molecular tests in cases of NB.

Keywords: Neuroblastoma, MYCN, molecular heterogeneity

ÖZ

Amaç: Tümör heterojenitesi, aynı tümör numunesindeki kanser hücreleri arasındaki farklılıkları tanımlar. Nöroblastom (NB), tümör heterojenitesinin tedavisini zorlaştırdığı bir kanser türüdür. Bu çalışma, NB'de rutin moleküler testlerle tespit edilen moleküler heterojenliğin rolünü araştırmayı amaçlamaktadır.

Yöntem: Çalışmaya 71 hasta dahil edildi. NB örnekleri 2012-2020 yılları arasında moleküler testlerle değerlendirilen 1.300 NB örneği arasından Türk Pediatrik Onkoloji Grubu Protokolü yönergelerine göre seçildi. Tümörün iki farklı bölgesinden (senkron) veya iki farklı zamanda (metakron) alınan moleküler araştırmalar (toplam 142 örnek) yapıldı. Heterojenite beş test için sorgulandı: MYCN amplifikasyonu, 1p36LOH, 11q23 silme ve 17q25 kazancı (gerçek zamanlı PCR ile tanımlandı) ve DNA ploidi (akış sitometrisi ile tanımlandı).

Bulgular: MYCN için %22,53, 11q23del için %29,58, 1p36LOH için %36,62 ve 17q25 kazancı için %40,85 oranında heterojenite gözlenirken, DNA ploidisi %36,4 oranında heterojendi. Moleküler heterojenite, metakron ve senkron olgular arasında istatistiksel olarak farklılık göstermedi. Yüksek riskli olgularda her iki grup arasında anlamlı bir fark olmaksızın daha sık moleküler heterojenite sergilemiştir.

Sonuç: Bulgularımız, moleküler heterojenitenin bir tümörün farklı bölgelerinde var olduğunu veya aynı tümörde farklı zamanlarda görüldüğünü desteklemektedir. Tümör doku örneklerinde birden fazla moleküler analiz yapılması faydalı olacaktır. Ek olarak, metakron metastazlardan nüksler veya yeniden biyopsi örnekleri, NB olgularında moleküler testler kullanılarak yeniden değerlendirilmelidir.

Anahtar kelimeler: Nöroblastom, MYCN, moleküler heterojenite

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INTRODUCTION

Cancer heterogeneity refers to the presence of distinct biological features in various sections of the tumor, attributed to tumor cells exhibiting diverse characteristics[1-3]. In metachronous heterogeneity samples 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. In synchronous heterogeneity, samples taken from different regions of the same tumor display different biological features[4]. Treatment 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 targeted therapies is considered to be biological heterogeneity[6,7]. As a result, identification of different clones in targeted treatment and mapping these clones has gained importance in molecular treatment.

Neuroblastoma (NB) shows pronounced heterogeneity[8] which complicates the treatment of NB[9]. Contrary to good survival in the low and moderate risk groups, treatment efficacy is limited in the high-risk group. In Turkey, survival rates for NB are 100%, 75.8%, 34.1%, 6.5% and 59.4% for stage 1, 2, 3, 4 and 4S, respectively[10,11]. Turkish Pediatric Oncology Group (TPOG) NB data from 2009 shows that in the low-risk group the three-year overall survival (OS), and event free survival (EFS) rates were 98%, and 90%, respectively. In the moderate risk favourable histology group, three-year OS, and EFS were 100% and 93%, respectively. In the moderate risk unfavourable histology group, three-year OS, and EFS were 90% and 76%, respectively. In the high-risk group, conventional CT branch had three-year OS of 53% and EFS of 37%, whereas the high-dose computed tomography (CT) branch had OS of 59% and EFS of 33%[12]. Though NB contains a variety of molecular targets like ALK and GD-2[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 NB, FISH and qPCR methods are commonly used in heterogeneity studies. Currently, guidelines recommend performing two molecular studies in at least two areas of the tumor, if macroscopic areas from NB display different colors[18]. Though the presence of *MYCN* with molecular heterogeneity is known for a long time, clinical implications of this heterogeneity has not been clarified yet[16].

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

MATERIALS and METHODS

Patient and Samples

Our research was approved by the Ethics Committee of Dokuz Eylül University Non-Interventional Research Ethics Committee (decision no: 2018/05-21, date: 15.02.2018) and patient relatives signed an informed consent form. The universe of the study comprised 1,300 fresh or paraffin-embedded tumor tissue samples sent to us between 2012-2020 for molecular evaluation according to the TPOG 2009 NB protocol. Two samples taken from the same patient for molecular investigation were included in two groups. The metachronous group (n=37) comprised all patients who gave more than one sample at least two months apart (a total of 1,300 samples, and 1,263 cases). The synchronous group (n=34) comprised randomized patients with enough tissue on surgical specimens retrieved from different areas of differentiation. Samples were chosen from differently colored macroscopic areas of the tumor. For the synchronous group, the first sample was taken from less differentiated dark-colored areas, the most undifferentiated blastic area (sample A), while the second sample was taken from the most differentiated area, which matches to light-colored areas in paraffin-embedded blocks (sample B). The clinical data for 71 patients included in the study (37 double sample metachronous group, 34 double sample synchronous group) were recorded from the patient medical files.

Molecular Investigations

Real time PCR and DNA ploidy method have been described in our previous studies[19].

DNA Isolation

DNA isolation was performed using a PCR (Roche® High Pure PCR Template Preparation Kit) kit according to manufacturer's guidelines. Fresh tumor tissue samples were imprinted on a slide, stained with toluidine blue and examined under a light microscope. Tumor tissue samples each 3 mm in diameter were used. After mechanical disintegration of tissue samples, they were left in proteinase K and lysis buffer for 2 hours at 55 °C. Paraffin-embedded samples were left in proteinase K and tissue lysis buffer overnight at 37 °C. The DNA samples were isolated according to manufacturer's guidelines. Quantification was assessed with Qubit® fluorometer.

Real-Time PCR

DNA obtained from tumor tissues were assessed in terms of *MYCN* amplification, 11q23 deletion, 1p36 LOH, and 17q25 gain with real-time PCR tests. Identification of abberations in these regions were assessed using labeled probes designed for these regions. The properties of genes are listed in Table 1.

The primer pairs, TaqMan probe, enzyme mixture were used for Real time PCR tests. As control DNA normal DNA not containing these aberrations (custom standard DNA) was used. Results were calculated as target/control proportional values and delta Ct method was used to assess rates of gene expressions 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 value and results were recorded as positive or negative.

DNA Ploidy

The transfer solution (containing tumor cells after tissue was removed) was centrifuged at 2,000 rpm for 7 minutes, then tissue medium cells obtained were suspended in freeze media (95% RPMI complete solution (88% RPMI+10% Fetal Bovine Serum +1% L-glutamine+1% penicillin-streptomycin) +5% DMSO) for assessment of DNA index. These cells were stored at -20 °C until DNA index analysis. DNA index was identified with BD Accuri™ C6 cytometer using cell cycle kit. Flow cytometry analyzes light radiated at 564 nm and 606 nm by cells with stained routinely with propidium

Table 1. The Properties of real time PCR design

Gene	MYCN	GNB1 (guanine nucleotide binding protein (G protein))	ARCN1 (archain 1)	Survivin
Location	2p24.3	1p36.33	11q23.3	17q25
Mutation, $2^{-\Delta\Delta Ct}$	Amplification, >10	Deletion, <0.5	Deletion, <0.5	Gain, >1.3
Primer Pair	5'GTGCTCTCCAATTCTCGCCT-3' 5'-GATGGCCTAGAGGAGGGCT-3'	5'-AGCCAGTGGCAAATGCATT-3' 5'TCTCTGCAGCCCTACCATTGA-3'	5'- ATCTGGAGGCAGCACAGCT-3' 5'- TACACTGGATTATACCTGGCTGG-3'	5'-GGGCTGCCACGTCCAC-3' 5'-GTCGTCATCTGGCTCCCA-3'
Taqman Probe	'5'FAM-CACTAAAGTTCCTT CCACCCTCTCCT-TAMRA-3'	'5'-FAMAGCAAATCAAGACA TCATGTAAACGCTCA- BBQ(tamra)'	'5'-FAM- CCATGATCACAG AGACCATCATTGAAA-BBQ(tamra)	'5'FAM-TTCATCCACTGCCC CACTGAGAACGA-TAMRA-3'
Reference Gene, Location	NAGK (n-acetylglucosamine kinase) 2p13.3	NGFB (nerve growth factor (beta polypeptide) 1p13.1	MYBPC3(myosin binding protein C cardiac) 11p11.2	TP53 17p13.1
Reference Gene Primer Pair	5'-TGGGCAGACACATCGTAGCA-3' 5'-CACCTTCACTCCCACCTCAAC-3'	5'- TTCTATCCTGGCCACACTGAG-3' 5'- TTCTGACTTGCCCTCTCAGGT-3'	5'-TGGTGTACGAGATGCGCGTC-3' 5'TCACCGATAGGCATGAAGGG-3'	5'TGTCCTTCCTGGAGCGATCT-3' 5'-CAAACCCCTGGTTTAGCACTTC -3'
Reference Gene Taqman Probe	'5'-VIC-TGTTGCCCGAGAT TGACCCCGT-TAMRA-3' '	5'-FAM(VIC)- TTG CCA AGG TCC TCC CTC TGC AGC T-BBQ (tamra)	'5'-FAM(VIC)- TCAACGCCATC GGCATGTCCAGG-BBQ(tamra)	'5'-(FAM) Yakima yellow-CAGC CCCCGGCTCCGCTAGA-TAMRA-3'

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 calculated by dividing relative DNA content mode in the sample G0/G1 population by relative DNA content in the control G0/G1 population. Additionally, variation coefficients are given for each G0/G1 peak.

Statistical Analysis

Findings were analyzed using SPSS 22.0. After descriptive statistics, cases were assessed for the presence of heterogeneity. The parameters were tested with Kolmogorov-Smirnov test under normality plots. Chi-square or Fisher's exact test were done as needed to the smallest groups and two-element parameters. Age was in normal Q-Q plot with p=0.0001. After normal distribution tests non-parametric (chi-square, Mann-Whitney U tests) or parametric tests (t-test) (for normal distribution) were used for statistical analysis.

RESULTS

In total, 71 double samples (142 samples from 71 patients) were obtained in this study from 71 cases. The metachronous group comprised 37 cases, while synchronous group comprised 34 cases. The cases comprised 35 girls and 36 boys. The metachronous group comprised 6 low, 5 intermediate and 26 high-risk cases, while the synchronous group comprised 14 low, 7 intermediate and 13 high-risk cases. The clinical and molecular features are present in Table 2.

Heterogeneities regarding MYCN (22.53%), 1pLOH (36.62%), 11qdel (29.58%), 17qGain (40.85%) were detected at indicated rates. 36.4% of the cases was heterogeneous on DNA ploidy. Based on patients' clinical data, 58.5% of cases had metastatic disease and 32.7% of them had relapse. Among patients with available survival information, 67.3% (33/49) had disease-free survival, 8.5% had survival with disease and 22.4% died. In total, 6 cases had risk difference between samples. Two of these 6 cases were diagnosed with Stage 4S at the time of diagnosis, which was later corrected to stage 4. One case was refractory to treatment so transferred from the moderate-risk unfavorable histology group to high-risk group. Three cases had variations in risk groups due to heterogeneity. All these cases were negative for *MYCN* with risk class variations due to heterogeneity observed in terms of 1pLOH, 11qdel and 17qGain. All cases with *MYCN* heterogeneity were in the high-risk group (Table 3).

In our study, the case coded 18 had a unique characteristic and so it is appropriate to present relevant findings independently. The female patient aged 12 months and 1 week was assessed in the low-risk group due to being stage 2B at the time of diagnosis but she 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 time: 18 months 1 week). Two tissue samples were assessed with Shimada classification as unfavourable histology. Firstly tru-cut (NB407) biopsy samples, and 3-days later resection

Table 2. Distribution of metachronous and synchronous cases according to molecular heterogeneity. Molecular heterogeneity did not show statistical difference among metachronous and synchronous cases. In both groups high-risk cases more often showed molecular heterogeneity

	MYCN heteroGENEITY	**1pLOH heteroGENEITY**	**11q Del heteroGENEITY**	**17q 25 Gain heteroGENEITY**
MetaCHRONous	27.02% (10/37) Low risk: 0/6 Intermediate risk: 0/5 High risk: 10/26	40.54% (15/37) Low risk: 1/6 Intermediate risk: 3/5 High risk: 10/26	21.62% (8/37) Low risk: 0/6 Intermediate risk: 1/5 High risk: 7/26	45.95% (17/37) Low risk: 1/6 Intermediate risk: 2/5 High risk: 17/26
sYNCHRONous	17.65% (6/34) Low risk: 1/14 Intermediate risk: 0/7 High risk: 5/13	35.29% (13/34) Low risk: 6/14 Intermediate risk: 1/7 High risk: 3/13	38.23% (13/34) Low risk: 6/14 Intermediate risk: 1/7 High risk: 6/13	32.35% (11/34) Low risk: 4/14 Intermediate risk: 1/7 High risk: 4/13
Total	22.53% (16/71) Low risk: 1/20 Intermediate risk: 0/12 High risk: 15/39	36.62% (26/71) Low risk: 7/20 Intermediate risk: 4/12 High risk: 13/39	29.58% (21/71) Low risk: 6/20 Intermediate risk: 2/12 High risk: 13/39	40.85% (29/71) Low risk: 5/20 Intermediate risk: 3/12 High risk: 21/39
P-value chi-square	0.7	0.07	0.54	0.277

material (NB413) were sent to us. Samples were assessed in the synchronous group, and any differences in risk groups could not be found in samples coded NB407 and NB413 as for gene expressions studied [*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 had *MYCN* amplification (139 times) with 11qdel- negativity, 17qGain- negativity and 1pLOH could not be studied. This patient would be assessed as low-risk group according to TPOG-2009 NB protocol (19); however, the *MYCN* amplification in the third tissue sample obtained 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 frequencies of metastases. Chi-square test could not reveal any correlation between metachronous-synchronous groups and heterogeneity, risk differences and frequencies of metastases. Though high-risk cases more frequently showed molecular heterogeneities, the Fisher exact test could detect any statistically significance difference between both groups (p=0.190).

DISCUSSION

In this study, molecular investigation was performed in tumor tissue samples taken from two different areas of tumor samples were examined prospectively at time of diagnosis (synchronous) or at two different time points (metachronous) to investigate heterogeneity in different samples of NB cases. Our study used PCR for molecular investigation instead of the FISH method used in the literature and rates of heterogeneity were compared with RT-PCR findings. Though the presence of *MYCN* heterogeneous cases is known in NB[17,20,21], studies about other genetic markers are limited in number. In our study, 1pLOH, 11qdel and 17qGain heterogeneity was observed with high frequency and this heterogeneity may affect clinical decision-making process.

In the literature, the percentage of heterogeneity for *MYCN* amplification identified with the FISH method in NB patients is above 40%[22]. In our study, we used PCR method instead of FISH method for identification of heterogeneity and our lower heterogeneity rates might be due to our preference for PCR.

Marrano et al.[17] reported a series of 30 cases where 102 tumor tissue specimens were examined for *MYCN* amplification to reveal heterogeneity. They studied metachronous tissue samples for comparison, evaluated *MYCN* status before and after treatment, and showed changes in *MYCN* expression in 20 cases. *MYCN* copy number was reduced in nine cases. Focal MYCN amplification was observed in five cases that had initially shown diffuse MYCN amplification. Conversely, two cases initially exhibiting focal MYCN amplification transitioned to diffuse MYCN amplification. Furthermore, one case underwent a change from diffuse MYCN amplification to MYCN gain. Additionally, three cases initially demonstrating focal amplification in the first specimen later tested negative for MYCN amplification. When we compare their findings with ours, although we used a different method for evaluation of our data, greater number of cases included in our study, and investigation of both synchronous and metachronous cases constitute strengths of our study We also studied

Table 3. Clinical properties of the cases. The cases with double samples in both metachronous and synchronous groups are similar in distribution

	n	Mean age months	Sex M: n F: n	MycN amp	1p LOH	11q del	17q gain	Risk class
Metachronous	37	38.81±40.34 (0-192) Median: 30	M: 17 F: 20	7 18.9%	14 37.8%	10 27%	15 40.5%	Low risk: 6 Intermediate risk: 5 High risk: 26
Synchronous	34	38.09±44.86 (2-192) Median: 18	M: 19 F: 15	5 14.7%	14 41.2%	10 29.4%	17 50%	Low risk: 14 Intermediate risk: 7 High risk: 13
Total	71	38.47±42.26 (0-192) Median: 24	M: 36 F: 35	12 16.9%	28 39.4%	20 28.2%	32 45.1%	Low risk: 20 Intermediate risk: 12 High risk: 39

M: Male, F: Female

molecular heterogeneity in other parameters including 1p36LOH, 11q23 deletion, 17q25 gain and DNA ploidy.

Study Limitations

Heterogeneity in metachronous cases of NB should be determined. This point of view may be helpful in approaching to molecular evaluation of NB from a different perspective. The main cause of samples sent from clinics being recollected and resent is sample insufficiency, which is the main limitation due to very scarce amount of data for dependent group assessment of patients included in our study. In clinical practice, oncology patients are not always available for rebiopsy at relapse and/or treatment-refractory conditions.

Investigation of specific genes in chromosomal regions using more specific genetic markers for heterogeneity in NB may ensure more effective research about the significance of heterogeneity in NB especially for next-generation sequencing. In this way, an effective increase in rates of clinical prediction and more successful treatment planning for high-risk NB cases may be achieved[23-25].

CONCLUSION

Based on our results we have concluded that there is molecular heterogeneity for *MYCN* amplification, 1p36LOH, 11q23 deletion, 17q25 gain and DNA ploidy in NB. It will be beneficial to perform molecular studies more than once on several tissues in NB cases. Our findings suggest that it will be beneficial to perform molecular analyses by sampling as much tumor as possible in cases where full response could not be achieved from the treatment of recurrence(s) or treatments applied at different time periods during the clinical course of NB. As number of studies on liquid biopsy or bone marrow aspiration increase, more clarity will be gained about whether these applications can take the place of tumor tissue biopsy.

Ethics

Ethics Committee Approval: Our research was approved by the Ethics Committee of Dokuz Eylül University Non-Interventional Research Ethics Committee (decision no: 2018/05-21, date: 15.02.2018).

Informed Consent: Patient relatives signed informed consent forms.

Peer-review: Externally peer reviewed.

Author Contributions

Surgical and Medical Practices: T.Ç.A., S.A., Concept: T.Ç.A., S.A., Design: T.Ç.A., S.A., P.E., N.O., Data Collection or Processing: T.Ç.A., S.A., E.Ö.S., P.E., M.A., Ö.E.G., A.E., Analysis or Interpretation: T.Ç.A., Literature Search: T.Ç.A., S.A., E.Ö.S., Z.A., N.O., Writing: T.Ç.A., S.A., E.Ö.S., Z.A.

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