MAPK/ERK signal pathway alterations in patients with Langerhans Cell Histiocytosis

Změny v signální dráze MAPK/ERK u pacientů s histiocytózou Langerhansových buněk

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Summary

Background: Clinical outcomes of Langerhans cell histiocytosis (LCH) are highly variable. It has been suggested that mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) signaling pathway might be activated in LCH patients. ***Materials and Methods:*** We investigated *KRAS*, *BRAF* and *NRAS* mutations in patients with LCH by qPCR. ***Results:*** Eight adult patients with LCH were treated at the National Cancer Institute, Kiev, Ukraine. Five patients received chemo plus radiation therapy and three patients received only chemotherapy, resp. ($p < 0.05$). All patients received LCH–I study protocol, six cycles in average. A *BRAF* c.1799T > A, p. V600E mutation was detected in 25% (2/8) of cases – 1 patient had an early relapse in 6 months, and 1 patient – stable disease. We did not find any *BRAF*, *KRAS* or *NRAS* mutations in three patients with late relapses (in 15, 24 and 46 months). Notably, *KRAS* mutations were not revealed in any LCH samples. The *NRAS* c.182A > G, p. Q61R mutation was found in two cases – one patient had LCH transformed to Hodgkin's lymphoma, one patient had a refractory disease. Time to relapse rate (TTR) in patients with and without *BRAF V600E* gene mutation was 13 vs. 28 months, resp. ($p < 0.05$). TTR was 31.3 vs. 6.41 months in patients with absence and presence of *NRAS* mutation, $p < 0.05$. Multivariate analysis showed the presence of *NRAS Q61R* mutation was associated with poor event-free survival in LCH patients with HR of 6.1 (95% CI 0.2–12.6; $p = 0.008$). ***Conclusion:*** *BRAF* and *NRAS* mutations in LCH suggest a possibility of the disease being driven by the activation of the MAPK/ERK pathway. These oncogenic mutations provide new opportunities in understanding LCH pathogenesis and may be a potential target of therapy.

Key words

Langerhans cell histiocytosis – mutations – prognostic factors – relapse – survival

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Souhrn

Úvod: Klinické výstupy histiocytózy Langerhansových buněk (Langerhans cell histiocytosis – LCH) jsou vysoce variabilní. Bylo navrženo, že u pacientů s LCH může být aktivována signální dráha proteinkinázy aktivované mitogenem (MAPK)/extracelulární signální regulační kinázy (ERK). ***Materiál a metody:*** Vyšetřili jsme mutace *KRAS*, *BRAF* a *NRAS* u pacientů s LCH pomocí metody kvantitativní polymerázové řetězové reakce. ***Výsledky:*** Osm dospělých pacientů s LCH bylo léčeno v Národním onkologickém institutu v Kyjevě na Ukrajině. Pět pacientů dostalo chemoterapii plus radiační terapii a tři pacienti dostali pouze chemoterapii ($p < 0,05$). Všichni pacienti dostávali studijní protokol LCH-I, v průměru šest cyklů. *BRAF* c.1799T> A, mutace V600E byla detekována u 25 % (2/8) případů – jeden pacient měl časný relaps po 6 měsících a jeden pacient stabilní onemocnění. U tří pacientů s pozdními relapsy (v 15, 24 a 46 měsících) jsme nenašli mutace *BRAF*, *KRAS* ani *NRAS*. Je zajímavé, že mutace *KRAS* nebyly odhaleny u žádných vzorků LCH. *NRAS* c.182A> G, mutace Q61R byla nalezena ve dvou případech – jeden pacient měl LCH transformovaný na Hodgkinův lymfom, jeden pacient měl refrakterní onemocnění. Doba relapsu (time to relapse rate – TTR) u pacientů s mutací genu *BRAF* V600E a bez mutace byla 13 oproti 28 měsícům, resp. $p < 0,05$. TTR byl 31,3 oproti 6,41 měsícům u pacientů s absencí a přítomností mutace *NRAS*, $p < 0,05$. Multivariační analýza ukázala, že přítomnost *NRAS Q61R* mutace byla spojena se špatným přežíváním bez příhody u pacientů s LCH s HR 6,1 (95% CI 0,2–12,6; $p = 0,008$). ***Závěr:*** Mutace *BRAF* a *NRAS* u LCH naznačují možnost, že onemocnění je řízeno aktivací cesty MAPK/ERK. Tyto onkogenní mutace poskytují nové možnosti v porozumění patogenezi LCH a mohou se stát potenciální cílovou terapií.

Klíčová slova

histiocytóza Langerhansových buněk – mutace – prognostické faktory – relaps – přežití

Introduction

Langerhans cell histiocytosis (LCH) is a rare myeloproliferative disorder of unknown etiopathogenesis. Based on modern research, LCH is a disease of the immune system with an abnormal immune response, leading to the proliferation of Langerhans cells, eosinophilic infiltration, granuloma formation, fibrosis, osteolytic lesions, etc.

The age-adjusted incidence rate of LCH is 3–5 cases in children and 1–2 cases in adults per 1,000,000 per year, with the incidence rate higher in men than in women. The sex ratio (m : f) is 2 : 1.

Clinical symptoms in LCH patients are very different – from isolated bone lesions to multisystem disease, with outcomes ranging from spontaneous remission to progression during therapy. In addition, symptoms of LCH vary depending on the organ or system involved. Rapid progressive forms are often seen in children and usually not observed in adults [1].

Regardless to similarities in histological features between LCH lesions, clinical outcomes are highly variable and range from isolated skin or bone disease to highly aggressive subtypes with life-threatening multisystem lesions, which require intensive chemotherapy.

There are different types of LCH (stratification of LCH):

1. multisystem LCH (MS-LCH) – with or without the involvement of ''risk organs";
2. single system LCH (SS-LCH) – unifocal or multifocal lesions;
3. single system LCH (SS-LCH) – with "special site" lesions

There are no universal guidelines for the diagnosis and treatment of adult LCH patients. The largest number of patients was analyzed in a retrospective pooled analysis from several national registries [2,3]. In general, physicians should have an option to choose the course of chemotherapy treatment depending on the stratification of LCH and presence of genetic mutations.

The benign morphology of LCH proliferating cells and their characteristic inflammatory infiltrates suggest that LCH may be an inflammatory disorder [4]. Dysregulated expression of inflammatory cytokines, such as interleukin-17A, has been reported [5]. However, the pathologic Langerhans cells in LCH are clonal [6]. Although clonality is an important feature of neoplasia, recurrent genomic abnormalities will be required to demonstrate that LCH is a neoplasm; until now, none of them have been reported [7].

It is known that the mitogen-activated protein kinase (MAPK) pathway is constantly activated in LCH. Mutations of the downstream kinases *BRAF* and *MAP2K1* mediate this activation in a subset of LCH lesions. The most common missense mutation of *BRAF* (mainly *V600E*) contributes to the incidence of various types of cancer, including LCH. *BRAF* gene located on chromosome 7q34 encodes a cytoplasmic serine-threonine kinase. This mutated BRAF protein constitutively activates the MAPK signaling pathway, which results in increased cell proliferation, apoptosis resistance and tumor progression [8,9].

Specific inhibition of *BRAF* signaling is effective in blocking proliferation of melanoma cells that have additional genomic abnormalities. Recent identification of cancer-associated mutation *BRAF V600E* in LCH cases provided molecular evidence of the neoplastic nature of LCH [10]. Initially *BRAF* was discovered in other types of cancer; additionally, it was found in LCH. Based on such data, it is possible to suggest that melanoma treatment approaches can be used for LCH treatment taking in consideration the expression of this gene [11]. *BRAF* mutations are usually found in tumors that have wild-type *KRAS* and *NRAS*, *KIT*, and other driver mutations.

Initially, LCH was considered as a disorder of immune regulation. Activating mutations in the proto-oncogene *BRAF-V600E* were reported in approximately 50–60% of cases; mitogen-activated protein kinase (MEK) and extracellular signal-related pathway (ERK) phosphorylation was reported in 100% of these cases. These data allow to relate LCH to a dendritic cell neoplasm with a strong inflammatory component. Current international LCH trials are focused

Tab. 1. Primer and probe sequences for analysis of qPCR mutations.

Target	Primer	Sequence
KRAS	common primer A	GTA CTG GTG GAG TAT TTG ATG TGT ATT AAC C
	probe 1	VIC-CTA CCA CAA GTT TAT ATT CAG TCA TTT TCA-TAMRA
	G12V	TAT CGT CAA GGC ACT CTT GCC TAC GCC TA
	G12D	TAT CGT CAA GGC ACT CTT GCC TAC GCC TT
	G12A	TAT CGT CAA GGC ACT CTT GCC TAC GCC TG
	K13A	CGT GTA TCG TCA AGG CACTCTTGC CTA CCT
	common primer B	CTC ATG AAA ATG GTC AGA GAA ACC TTT ATC
	Probe-2	6-FAM-CAA GAG TGC CTT GAC GAT ACA GCT A-TAMRA
	G12C	CTG AAT ATA AAC TTG TGG TAG TTG GAG CAT
	G12S	CTG AAT ATA AAC TTG TGG TAG TTG GAG CCA
	G12R	CTG AAT ATA AAC TTG TGG TAG TTG GAG CCC
β-actin	forward	TCA CCC ACA CTG TGC CCA TCT ACG A
	reverse	CAG CGG AAC CGC TCA TTG CCA ATG G
	probe	FAM-ATG CCC TCC CCC ATG CCA TCC TGC GT
BRAF	forward	CTG TTT TCC TTT ACT TAC TAC ACC TCA GAT
	reverse (mutant V600E)	CCC ACT CCA TCG AGA TTT CT
	reverse (reference)	CAA CTG TTC AAA CTG ATG GG
	probe	FAM-CAC AGT AAA AAT AGG TGA T-MGB
CYP17	forward	CCC TAG AGT TGC CAC AGC
	reverse	GGT AAG CAG CAA GAG AGC
	probe	VIC-CTG TCT ATC TTG CCT GCC-MGB

qPCR – quantitative polymerase chain reaction

on further improving the outcome of patients with high-risk multisystem LCH, by decreasing the reactivation rate, optimizing early salvage regimens and preventing late complications.

Somatic mutations in *ARAF* and *MAP2K1* were recently discovered; these mutations lead to activation of the RAS-RAF--MEK-ERK pathway in the setting of wild--type *BRAF*. It was also found that the activation of mutation in *MAP2K1* is relatively insensitive to MEK inhibitors. This suggests that a more detailed understanding of this pathway in LCH may be necessary for the development of more efficient targeted therapies [12].

Materials and Methods

We performed the study from February 1, 2009 to March 31, 2017 on biopsy samples received at the Department of Onchematology of National Cancer Institute, Kiev, Ukraine. Altogether, 8 patients with LCH (6 males and 2 females; median age – 25, age range – 21–55) were included in this study. The diagnosis was based on clinical symptoms and immunohistochemistry results (CD1a+ or CD207+ and S-100+).

The study was approved by the institutional ethics committee review board at the National Cancer Institute of Ukraine.

Quantitative real-time PCR

All biomaterial was obtained before treatment. Fresh tumor samples for quantitative polymerase chain reaction (qPCR) analysis were stored in 'RNA-later' (Ambion, USA) to stabilize nucleic acids.

DNA was extracted from formalin-fixed, paraffin-embedded tissues after histological control. Genomic DNA from tumor samples was isolated using NucleoSpin Tissue Kit (Macherey-Nagel, Germany). Primers and probes were specially engineered to determine *KRAS* tumor genotype (7 mutations located within codons 12 (6) and 13 (1)) by the multiplexed qPCR. Sequences of primers were experimentally selected with Primer Express Software v3.0 (Applied Biosystems, USA) and synthesized by Applied Biosystems, USA. All primer and probe sequences are listed in Tab. 1.

For *KRAS* mutation analysis, primers and TaqMan probes were used at 20 μM and 5 μM concentrations, resp. TaqMan Universal PCR Master Mix (Applied Biosystems, USA). Common primer A, mutation-specific primer and probe 1 were used for K*RAS G12V, G12D, G12A, K13A* mutation analysis, common primer B, mutation-specific primer and probe 2 were used for *KRAS G12C, G1S, G12R* mutation analysis; 5–10 ng of DNA were used to prepare the reaction mix-

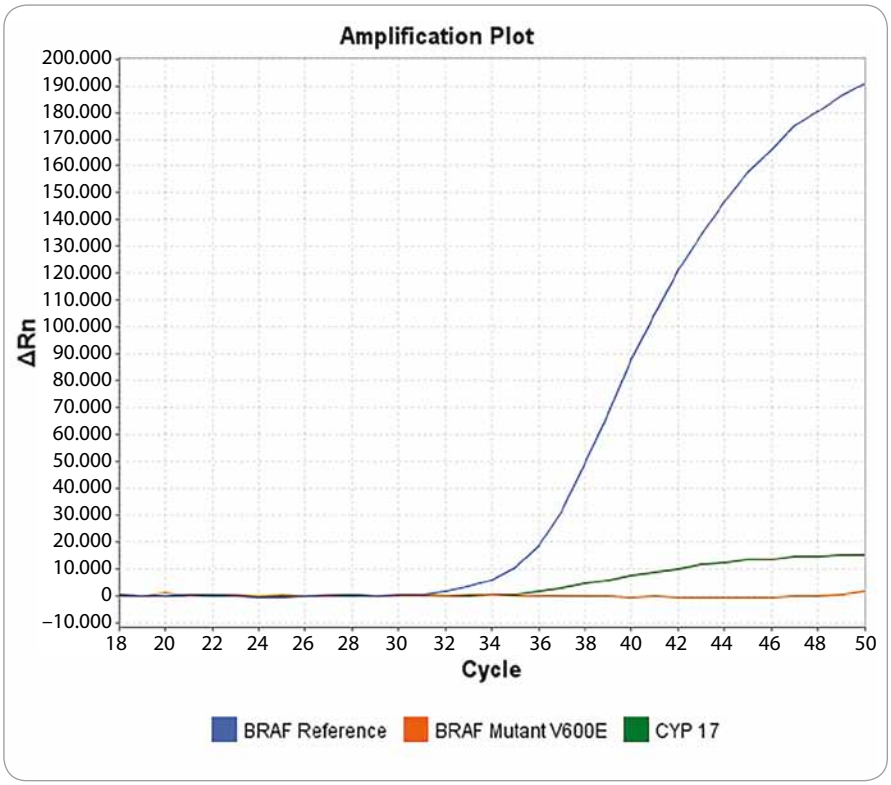
Tab. 2. Response to treatment.

Type of response	n
stable disease	5
partial response	1
complete response	1
relapse/refractory	1

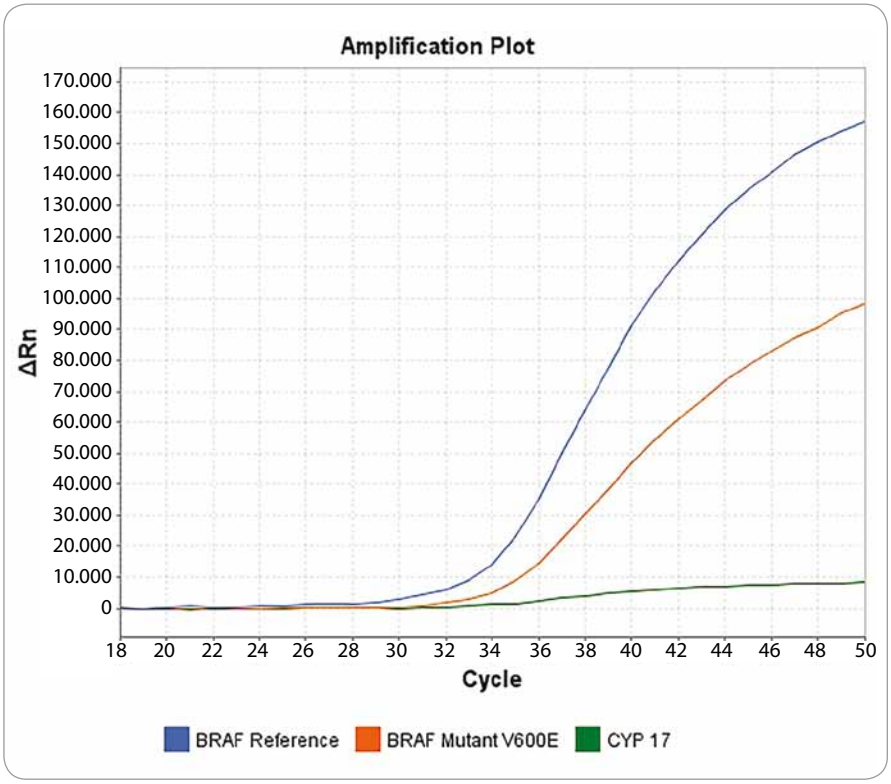
ture in a total volume of 25 µl. A total of 55 cycles of qPCR (95°C – 42 seconds, 60°C – 42 sec and 72°C – 52 sec) were run on 7,300/7,500 Real-Time PCR Systems (Applied Biosystems, USA). This assay uses six primers, two common primers, two TaqMan probes for seven different *KRAS* mutations. qPCR for an inner control β-actin was performed for each sample.

For *BRAF* mutation analysis qPCR reference PCR was performed in a 25 mkl reaction volume with 1 mkl TaqMan Universal Master Mix (Applied Biosystems, USA), 900 nmol/L of each *BRAF* mutation-unspecific primer, 100 nmol/L of the *BRAF* probe, 112.5 nmol/L of each internal control primer, 25 nmol/L of the internal control probe, and 5–10 ng of DNA of varying concentrations. Allele-specific PCRs were performed according to the same protocol but using a concentration of 450 nmol/L of allele-specific primer [13]. All real-time PCRs were performed on 7,300/7,500 Real-Time PCR Systems (Applied Biosystems, USA) under the following thermocycling conditions – 95°C for 10 min, followed by 50 cycles of 90°C for 15 sec and 60°C for 1 min. Cycle threshold (Ct) values were recorded for reference PCR and for each allele-specific PCR, and corresponding Ct values (ie, allele-specific Ct minus reference Ct) were calculated.

We also studied *KRAS* minor mutations *G13C, Q61R, Q61H, A146T and NRAS* mutations *G12V, G12D, G12C, G12S, G13V, G13R, Q61K, Q61L, Q61R, Q61HC, Q61HT* in tumor samples with TaqMan Mutation Detection Assays (Cat. #4465804, Applied Biosystems, USA) and a mutation-unspecific region was used as a reference amplicon (Cat. #4465807, Applied Biosystems, USA).



Graph 1. Case 1 SS-LCH with the involvement of bone system, identified by qPCR, no *BRAF V600E* mutation found.
SS-LCH – single sytem Langerhans cell histocystis, qPCR – quantitative polymerase chain reaction



Graph 2. Case 7 MS-LSH with the involvement of "special sites", identified by qPCR, with a *BRAF V600E* mutation.
MS-LCH – multisystem Langerhans cell histocystis, qPCR – quantitative polymerase chain reaction

Statistical analysis

A Spearman correlation coefficient (r) test and Cox test were used to determine the relationship between two continuous measurements. We used Chi-square tests for comparison of the time to relapse (TTR); $p < 0.05$ was considered to indicate a statistically significant difference. Survival curves were generated by the Kaplan-Meier method. An overall survival (OS) was calculated from the date of pathological diagnosis to the death or to last follow-up. An event-free survival (EFS) was calculated from the first day of treatment to relapse, progression or death, or to last date of follow-up. All the analyses were carried out using Statistica 10 and MedClac Version 12.6.1.0.

Results

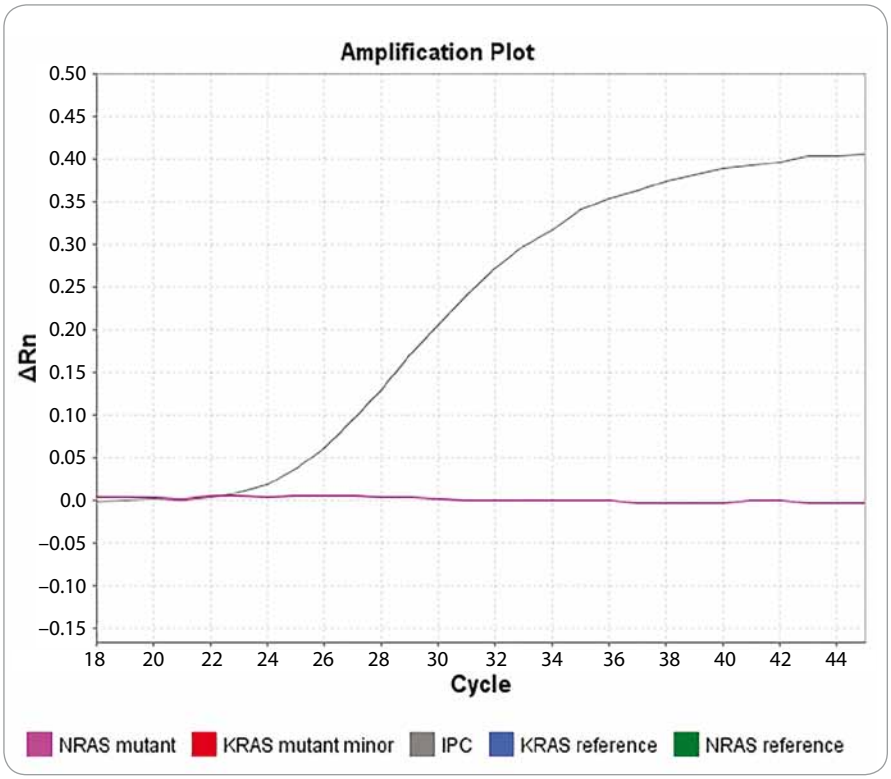
Single-system disease (SS-LCH) and multi-system LCH (MS-LCH) had 25% (2/8) and 75% (6/8) of patients, resp. ($p < 0.05$).

In two cases, SS-LCH patients had bone involvement and one patient had pulmonary system involvement. The patient with primary LCH of lungs had a long-term history of smoking (> 10 years).

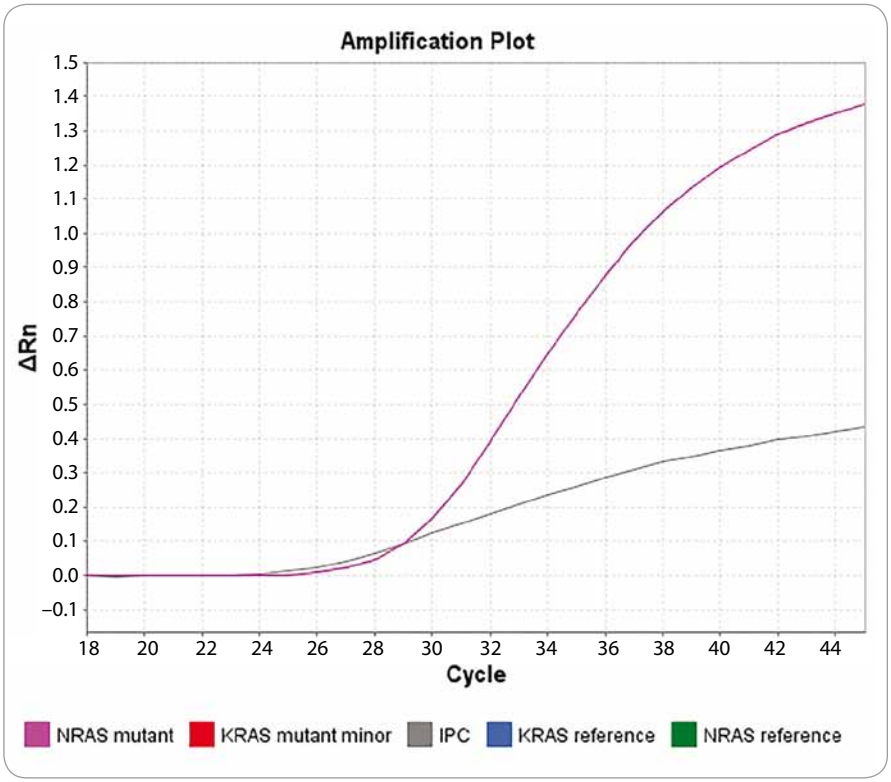
Two patients had MS-LCH with the involvement of "risk organs" such as central nervous system and bone marrow, there was only one patient with the involvement of "special sites". In addition, we diagnosed two cases of MS-LCH without the involvement of "risk organs". In our study, the patients older than 32 (50 and 55 years old) had MS-LCH with bone involvement, while patients younger than 24 (22 years old) had their pulmonary system involvement at diagnosis. A total of 62.5% patients received chemotherapy in combination with radiation therapy, and only 1/4 of patients (37.5%) received only chemotherapy, ($p < 0.05$). All patients received chemotherapy according to LCH-I protocol, six cycles on average (range 2–8).

The evaluation of response was done after the second or third cycle of chemotherapy. The response to treatment was achieved in 87.5% (7/8) of cases (Tab. 2.).

The maximum follow-up period in this group of patients was 96.72 months (median 44.92 months). A total of



Graph 3. Case 2 MS-LCH, with the involvement of risk organ, identified by qPCR, no *NRAS Q61R* mutation found.
MS-LCH – multisystem Langerhans cell histocystis, qPCR – quantitative polymerase chain reaction



Graph 4. Case 6 MS-LCH, without the involvement of risk organ, identified by qPCR, with a *NRAS Q61R* mutation.
MS-LCH – multisystem Langerhans cell histocystis, qPCR – quantitative polymerase chain reaction

Tab. 3. TTR based on disease stratification and *BRAF/KRAS/NRAS* gene mutations.

LCH stratification	Patients with disease progression, n	*BRAF/KRAS/NRAS* gene mutation	TTR (month)	Relapse type	Death
SS-LCH, multifocal > 1 bone	1	*BRAF V600E*	6	early	0
MS-LCH, without the involvement of "risk organ"	1	*NRAS Q61R*	3	refractory	0
MS-LCH, with the involvement of "risk organ"	1	*NRAS Q61R*	12	late	0
MS-LSH, with the involvement of "special sites"	0	*BRAF V600E*	0	0	0

TTR – time to relaps, LCH – Langerhans cell histiocytosis, MS-LCH – multisystem LCH, SS-LCH – single system LCH

87.5% (7/8) patients is still being followed up. During the follow-up period, there was one death registered due to the progression in LCH with pulmonary involvement.

There were 2/8 patients with early relapses (in 6 months), 2/8 patients with late relapses and 1/8 patient with refractory disease. In addition, one patient had transformed LCH into Hodgkin lymphoma, which was confirmed by immunohistochemical study.

Three-year EFS was 28.3%. It was impossible to calculate the OS due to the loss to follow-up of one patient. The analysis of data for presence of MAPK/ERK pathway gene mutations did not show any *BRAF/KRAS/NRAS* mutations in 50% (4/8) of patients.

We did not find any *BRAF, KRAS* or *NRAS* mutations in three patients with late relapses (in 15, 24 and 46 months). Notably, *KRAS* mutations were not revealed in any LCH samples.

A *BRAF* c.1799T>A, p. *V600E* mutation was present only in 2 out of 8 evaluated cases (25%):

- one patient with early relapse (in 6 months after the treatment);
- one patient with stable disease (Graph. 1 and 2)

We also identified two patients with *NRAS* c. 182A>G, p. Q61R mutation:

- one patient had LCH transformed to Hodgkin's lymphoma during 12 months after treatment LCH;
- one patient had a refractory disease (Graph 3 and 4.)

Due to a small number of patients in the research group, we decided to introduce TTR in months in order to be able to determine the impact of MAPK/EPK pathway mutation on relapse occurrence (Tab. 3).

We also analyzed correlation of disease response to the therapy, OS and EFS with age, sex, LCH stratification, number of cycles, response totherapy, radiation therapy, presence of gene mutations *BRAF/KRAS/NRAS*.

Unfortunately, due to a small number of patients, we did not identify a statistically significant difference of EFS in patients with or without *BRAF V600E* gene mutation. However, the comparison of patients with TTR with and without *BRAF V600E* gene mutation was significant, 13 vs. 28 months, resp.; $p < 0.05$.

In addition, TTR was 31.3 vs. 6.41 months in patients with the absence or presence of *NRAS* mutation, $p < 0.05$. Multivariate analysis confirmed that the presence of *NRAS Q61R* mutation has a significant association with shorter EFS in LCH patients with HR of 6.1 (95% CI 0.2–12.6; $p = 0.008$).

Discussion

LCH is a rare disease characterized by clonal proliferation of Langerhans cells [14]. Granuloma-like lesions of LCH have heterogeneous cellular composition. The etiology of Langerhans cell histiocytosis is largely unknown [15,16]. Recent studies have suggested that LCH is related to the clonal neoplastic proliferation of myeloid-derived precursor cells with a high frequency of somatic oncogenic *BRAF V600E* mutations in 25–60 % of LCH patients [17–20]. Since the follow-up time in our study for some patients was short, the relationship of *BRAF V600E* mutation to survival has not been sufficiently analyzed. Smoking is an important factor in primary LCH of lungs [20]; one patient with pulmonary disease had a long-term history of smoking in our study, which may be related to this form of LCH.

Symptoms, such as lytic bone lesions, exophthalmos, polyuria, hepatosplenomegaly, lymphadenopathy, skin rash, and hematological compromise, are most common in patients with LCH [21]. During our study, we most commonly came across such symptoms as lytic bone lesions (62.5%), lymphadenopathy (50%), polyuria (25%).

The overall 5-year survival rate of LCH patients is 88% [22]. Patients with unifocal LCH have an excellent prognosis and a high long-term survival rate (99%), may spontaneously recover or require minimal treatment [22]. In our cohort, one patient died because of pulmonary disease. The other seven patients are still alive, and further follow-up should be performed. There were no spontaneous recovery cases in our study.

The *BRAF V600E* mutation has been reported in 39–57% of Langerhans cell histiocytosis cases [17,19]. Although the pathophysiology of LCH remains unclear, activation of the MAPK/ERK signal pathway appears to play a significant role [23]. According to our data, *BRAF* gene mutation has been registered in only 25% of patients. The comparison of patients with TTR with and without *BRAF V600E* gene mutation was significant, 13 vs. 28 months, resp.; $p < 0.05$. Hypothetically, we can assume that the data analysis of a larger patient's cohort would be able to confirm the rela-

tion of the response to treatment with or without the expression of *BRAF V600E* gene mutation. *BRAF* gene can be used as a prognostic marker for the assessment of patient's response to treatment, receiving a standard chemotherapy for LCH as well as for patients participating in clinical trials.

The fact that some of LCH patients do not carry *BRAF* gene mutations prompts a possibility of other mutations that are partially or entirely related to ERK pathway and require further research. We did not find any *BRAF*, *KRAS* or *NRAS* mutations in three patients with late relapses.

Approximately 30% of LCH tumors have *MAP2K1* mutations. Somatic *MAP2K1* mutations have been identified in approximately 50% of *BRAF*– LCH samples [24]. In our study, there were also two *BRAF* patients who had a *NRASQ61K/R* somatic mutation. In addition, there are studies showing that the presence of concurrent *BRAF V600E* or *NRASQ61K/R* mutations was strongly associated with patient outcome [25]. Multivariate analysis in our cohort showed that presence *NRASQ61K/R* gene mutation has a significant association with poor clinical outcome in patients with LCH. Moreover, somatic mutations affecting other *BRAF* residues, as well as the *ARAF* gene, have recently been described [18,23].

The determination of the genetic mutation in LCH lesions has important implications for specific treatment. *BRAF* inhibitors, such as vemurafenib, have been successfully used to treat LCH patients with the V600E mutation [26]. The test for this mutation may play an important role for an individualized treatment. Additionally, a recent report on the successful use of BRAF protein inhibitor on refractory hairy cell leukemia [27] emphasized that LCH can potentially be treated with targeted therapy.

Conclusion

In summary, the presence or absence of one of MAPK/EPK pathway mutations, such as *BRAF, NRAS* or *KRAS* in a tumor, does not confirm or rule out a diagnosis of LCH. The results of these tests should be correlated with clinical findings and histopathologic features. Unfortunately, we did not find a strong significant impact of *BRAFV600E* gene mutation on clinical outcomes in patients with LCH in our study. However, our results provide evidence that *NRASQ61K/R* gene mutation can predict clinical outcome in patients with LCH and seem to be promising for the future studies.

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