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Epidermal Growth Factor Receptor immunohistochemical expression in hepatocellular carcinoma without Epidermal Growth Factor Receptor exons 18–21 mutations

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Introduction: EGFR targeted therapies, have been proved beneficial for patients with HCC, nevertheless additional research on EGFR immunoexpresion and EGFR mutations is still needed, especially in population in which it has not been done yet. The aim of this study is to evaluate EGFR immunoexpression in HCC without EGFR exons 18–21 mutations and to evaluate its influence on survival in HCC patients in North Macedonia.

Methods: We studied 31 cases of HCC for EGFR immunohistochemical expression and EGFR exons 18–21 mutations. The following clinical parameters were analyzed: Hepatitis B and C virus infection, presence of cirrhosis, tumor size, enlarged lymph nodes, metastases, alpha fetoprotein level and overall survival. Presence of the EGFR immunosignal (membranous and cytoplasmic) and the percentage of positive tumor cells in the entire tumor tissue specimen were semi-quantitatively determined.

Results: Hepatitis B and C virus infection, tumor size, metastatic disease and EGFR immunoexpression have influence on patient's survival. No EGFR exons 18–21 mutations were detected in this group of HCCs. EGFR expression of 61%–80% in tumor tissue significantly influenced survival of the patients ($p < 0.01$). Multiple Cox regression confirmed tumor size of 5–10 cm ($p < 0.05$), tumor size > 10 cm ($p < 0.01$) and EGFR expression in range of 61% to 80% ($p < 0.05$) as independent survival predictors in patients with HCC.

Conclusion: EGFR overexpression in range of 61% to 80% was an independent survival predictor in patients with HCC, implying that these patients could benefit from EGFR inhibition. However, the absence of EGFR mutations in exons 18–21 in any of the cases of this study suggest that single drug EGFR targeted therapy in patients with HCC may be insufficient.

Key words: Epidermal Growth Factor Receptor, hepatocellular carcinoma, survival, gene mutation, exon 18–21, immunohistochemistry, hepatitis B virus infection

What is new?

This is the first study in North Macedonia to analyze genes 18–21 in patients with HCC. Additionally, the study analyses immunohistochemical expression of EGFR in HCC without EGFR mutations in exons 18–21 and evaluates its influence on the survival of HCC patients in North Macedonia.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third cause of cancer-related mortality in the world [1, 2]. The prognosis of patients with HCC, except for those early diagnosed remains poor, despite many advances in HCC treatment [3]. Many patients diagnosed in advanced stage do not belong in a group of curatives, and treatments like a surgical resection or transplantation are not an option, therefore, palliative therapy is the only possible alternative [4]. The situation is more complicated because of the carcinogenic background due to cirrhosis and Hepatitis B and C virus infections. Systemic chemotherapy and other therapeutic modalities are usually followed by post-treatment relapses, drug resistance and tumor progression [5–7]. Even after surgery and transplantation recurrence rate is very high [7].

It is considered that dysfunction of intracellular signaling pathways play an important role in development and progression of cancers, including HCC. The explanation and understanding

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of molecular mechanisms of carcinogenesis and signaling pathways make a base for target therapies [8]. The epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase receptor, upon ligand binding, activates many diverse signaling pathways, which play a role in cell proliferation, differentiation, and survival [9, 10]. Overexpression of EGFR is reported in many cancers including HCC and high expression is correlated to advanced stage and shorter survival [11, 12].

EGFR targeted therapies, monoclonal antibodies and small molecule tyrosine kinase inhibitors have given some benefits to patients with HCC [8], so additional research on EGFR immunoexpresion and EGFR mutations is still needed, especially in parts of the world where it has not been done yet.

The aim of our study was to analyze immunohistochemical expression of EGFR in HCC without EGFR mutations in exons 18–21 and to evaluate its influence on the survival of HCC patients in Republic of North Macedonia.

MATERIALS AND METHODS

Thirty one cases of HCC were investigated for EGFR immunohistochemical expression and EGFR mutations in exons 18–21 were analyzed.

We added 18 new cases to a cohort of 60 patients that had been analyzed for multiple clinical and pathological parameters as it was already reported [13]. Out of 78 patients, 31 cases were selected for this study, based on the data that they all had undergone surgical tumor resection, without any other therapy before surgery, and had adequate liver functional reserve in the time of operation with Child-Pugh score A and B.

The patients were followed up for 24 months from the time of initial diagnosis.

Serological tests for hepatitis B and C virus infections were performed in all patients.

Imaging modalities: ultrasound and/or computed tomography we used to determine the size of tumor nodes (< 3 cm; 3–5 cm; 5–10 cm; > 10 cm), the presence of cirrhosis and enlarged lymph nodes. Serum level of alpha-fetoprotein (AFP) was quantitatively determined using a commercial ELISA test.

Immunohistochemistry

Tumor differentiation, microvascular invasion, presence of cirrhosis and histological pattern were defined during pathological investigation. Immunohistochemical staining with an antibody against EGFR (Epidermal Growth Factor Receptor, Monoclonal Mouse, Anti-Human, Clone EGFR.25, Leica Biosystems, Novocastra™ Liquid, dilution 1:50) using Avidin-Biotin immunoperoxidase technique were made. For the visualization of the antigen-antibody reaction, LSAB and En-Vision kit from DAKO was used.

Presence of the membranous immunosignal (regardless of the signal intensity) and the percentage of positive tumor cells were evaluated. The percentage of the stained cells was semi-quantitatively determined in the whole tumor tissue sample at the slide on microscopic fields at x 200 magnification, after previous analysis of the cell membranes at magnification at x 400 magnification.

Additionally, we created five groups of patients according to the percentage of EGFR immunoexpression in the whole tissue samples: group I with samples showing EGFR immunoexpression in 20% of cells or less; group II with 21–40% positive cells; group III with 41%–60% positive cells; group IV with 61% – 80% positive cells and group V with 81%–100% positive cells in the tissue sample (Fig. 1).

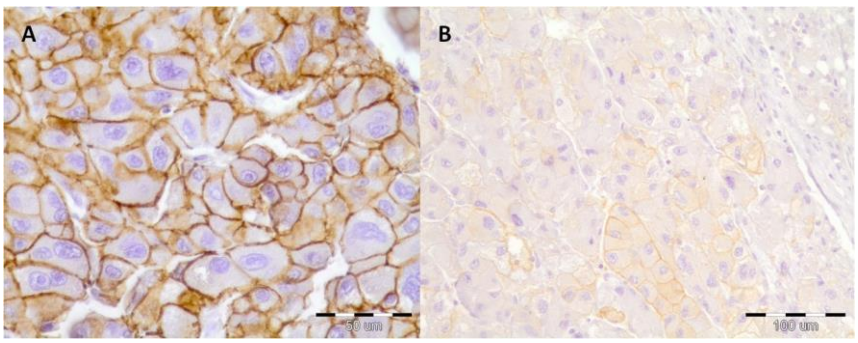


Fig. 1. Immunohistochemical expression of EGFR in HCC. A: In this field of the analyzed slide approximately 100% of cells show EGFR positivity with strong signal intensity (81%–100% group) (EGFR immunostaining x 400); B: Somewhat more than 40% of HCC cells show positivity in this microphotography with weak signal intensity (41%–60% group) (EGFR immunostaining x 200). Bar: um = μm (Place for figure 1)

In order to obtain objective findings of the EGFR immunoexpression, the slides were separately evaluated by two pathologists.

DNA isolation

DNA from paraffin embedded tissue (FFPE) was extracted using Cobas® DNA Sample Preparation Kit (Roche Diagnostics). DNA concentration was measured using ScanDrop2 (AnalyticJenna) spectrophotometer and dilution of isolated DNA was performed according to detecting mutations protocol.

Detection of EGFR mutations

Cobas® EGFR Mutation Test V2 (Roche Diagnostics) for detecting mutations in exons 18–21 of the EGFR gene was used. According the protocol, 2 ng/uL isolated DNA was used for detecting mutations with this assay on Cobas Z480 IVD RealTime PCR Machine. The Cobas® EGFR Test is designed to detect the following mutations:

Exon 18: G719X (G719A, G719C, and G719S)

Exon 19: deletions and complex mutations

Exon 20: S768I, T790M, and insertions

Exon 21: L858R and L861Q

A mutant control and negative control was included in each run to confirm the validity of the run. The Cobas® EGFR Test can detect mutations with at least 5% mutation level using the standard input of 50 ng per reaction well.

Statistical analysis

For statistical analysis we used statistical software package Statistica 7.1 for Windows and SPSS Statistics 23.0, applying: Descriptive Statistics, Kaplan Meier analysis (Log Rank (Mantel - Cox) & Breslow test and Cox regression (Wald / Exp (B) / 95.0% CI for Exp (B) /(p) / Backward Stepwise. Statistical significance was accepted when p-values < 0.05.

RESULTS

Out of 31 patients, 10 patients (31.67%) were female and 21 (67.74%) were male, with age ranging from 38 to 77 years, median 63.16 ± 86 years. Mean survival time for female patients was 13.30 ±3.05 months, for males 11.19 ±1.88 months, and overall survival was 11.87 ±1.62 months.

Twenty seven (87.09%) patients from this study had underlying cirrhosis. Twenty four patients (77%) had Child-Pugh score A and 7 patients (33%) had Child-Pugh score B.

Twenty nine patients (93.56%) had hepatitis B virus infection, one patient (3.22%) had hepatitis C virus infection and only 1 (3.22%) patient did not have hepatitis virus infection.

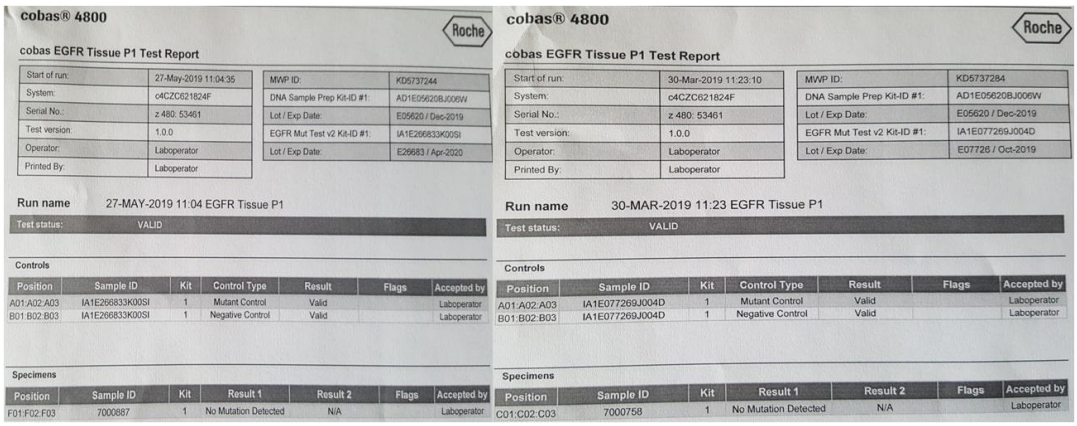
More than 1 centimeter enlarged lymph nodes were present in 4 (12.90%) patients out of 31 patients.

A disease recurrence developed in two patients (6.45%), and 5 (16.2%) patients developed extrahepatic metastatic disease during the follow up period.

Four patients (12.90%) had tumors with diameter 3 cm or less, 8 patients (25.81%) had tumors with dimensions from 3–5 cm, 12 (38.71%) patients had tumor with dimension 5–10 cm and 7 (22.58%) patients had tumors larger than 10 cm.

Alpha fetoprotein was in range of 1.84 to 4500 ng/mL, mean 691.2 4± 1120.73 ng/mL (normal range in adults 0–10 ng/mL).

No mutations were found in EGFR exons 18–21 in any of the cases of this study (Fig. 2).


cobas® 4800 — Roche

cobas EGFR Tissue P1 Test Report

Start of run:	27-May-2019 11:04:35	MWP ID:	KD5737244
System:	c4CZC621824F	DNA Sample Prep Kit-ID #1:	AD1E05620BJ006W
Serial No.:	z 480: 53461	Lot / Exp Date:	E05620 / Dec-2019
Test version:	1.0.0	EGFR Mut Test v2 Kit-ID #1:	IA1E266833K00SI
Operator:	Laboperator	Lot / Exp Date:	E26683 / Apr-2020
Printed By:	Laboperator		

Run name 27-MAY-2019 11:04 EGFR Tissue P1

Test status: VALID

Controls

Position	Sample ID	Kit	Control Type	Result	Flags	Accepted by
A01:A02:A03	IA1E266833K00SI	1	Mutant Control	Valid		Laboperator
B01:B02:B03	IA1E266833K00SI	1	Negative Control	Valid		Laboperator

Specimens

Position	Sample ID	Kit	Result 1	Result 2	Flags	Accepted by
F01:F02:F03	7000887	1	No Mutation Detected	N/A		Laboperator

cobas® 4800 — Roche

cobas EGFR Tissue P1 Test Report

Start of run:	30-Mar-2019 11:23:10	MWP ID:	KD5737284
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Serial No.:	z 480: 53461	Lot / Exp Date:	E05620 / Dec-2019
Test version:	1.0.0	EGFR Mut Test v2 Kit-ID #1:	IA1E077269J004D
Operator:	Laboperator	Lot / Exp Date:	E07726 / Oct-2019
Printed By:	Laboperator		

Run name 30-MAR-2019 11:23 EGFR Tissue P1

Test status: VALID

Controls

Position	Sample ID	Kit	Control Type	Result	Flags	Accepted by
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B01:B02:B03	IA1E077269J004D	1	Negative Control	Valid		Laboperator

Specimens

Position	Sample ID	Kit	Result 1	Result 2	Flags	Accepted by
C01:C02:C03	7000758	1	No Mutation Detected	N/A		Laboperator

Fig. 2. Cobas® EGFR Mutation Test V2. Results of two patients with HCC negative for EGFR exons 18–21 mutations.

Immunohistochemically EGFR positive cells in the tumor tissue samples ranged from 1% to 100%, with mean value of 46.9%. Distribution of patients according to the percentage of EGFR positive cells, number and percentage of lethal outcome are shown in Table 1.

In Table 2 mean and median time of survival are shown according to the percentage of EGFR positive cells i.e. according to the EGFR groups and in Fig. 3 Kaplan Meier curves are shown.

Table 1
Distribution of patients according to EGFR groups, number and percentage of lethal outcome

EGFR group	Total N	N of Events	Censored	
			N	Percent
< 20%	9	4	5	55.6%
21–40%	6	4	2	33.3%
41–60	3	3	0	0.0%
61–80%	8	8	0	0.0%
>81–100%	5	4	1	20.0%
Overall	31	23	8	25.8%

Table 2
Mean and median time of survival according to the percentage of EGFR positive cells

EGFR	Mean				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
< 20%	19.11	2.49	14.23	23.99	.	.	.	.
21–40%	11.50	3.89	3.87	19.13	8.00	6.12	00	20.00
41–60	10.33	5.61	00	21.32	8.00	4.90	00	17.60
61– 80%	5.75	1.82	2.18	9.32	3.00	1.41	23	5.77
81–100%	10.00	3.50	3.14	16.86	7.00	1.10	4.85	9.15
Overall	11.87	1.62	8.69	15.05	11.00	2.31	6.47	15.53

Estimation is limited to the largest survival time if it is censored.

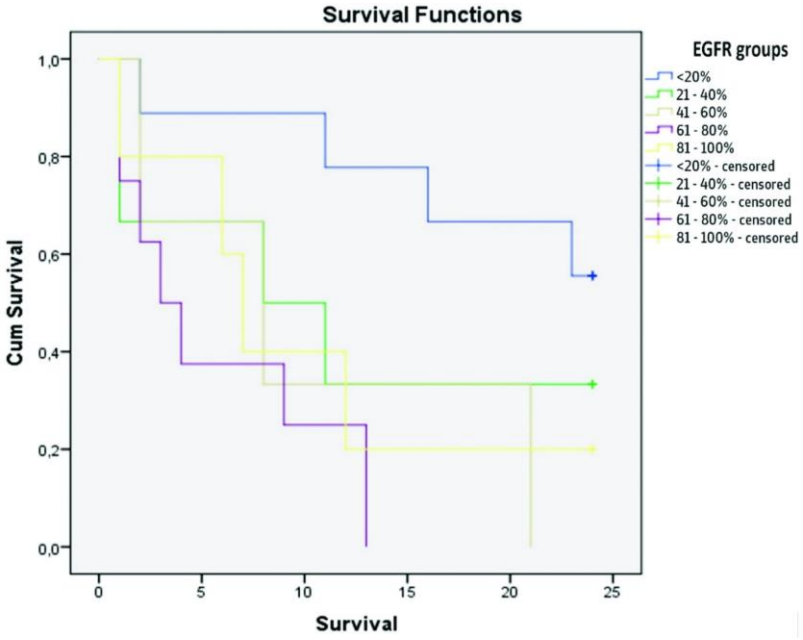

Fig. 3. Survival curves of the patients according to the EGFR positive cells group survival curves shows significantly shorter time of survival of patients whose HCCs expressed EGFR in range 61%–80% in comparison to HCCs expressing EGFR < 20%.

Statistically significant difference in survival time of patients whose tumors showed 61% to 80% EGFR positive cells in comparison to patients whose tumors presented 20% or less EGFR positive cells ($p < 0.01$; $p = 0.003$) was found.

Statistically significant difference of the survival rate for the presence of Hepatitis B and C virus infections ($p < 0.05$), tumor size ($p < 0.05$) and the presence of metastatic disease ($p < 0.05$) was also found. Patients with hepatitis B virus infection had significantly longer time of survival in comparison to patients with hepatitis C virus infection, patients with tumor size 5–10 cm and larger than 10 cm had significantly shorter time of survival than patients with tumors size < 3 cm, and patients with metastases had significantly shorter time of survival than patients without metastases.

Multiple Cox regression analysis including the variables which showed statistical significance in survival time in Kaplan Meier analysis (Hepatitis B virus infection, tumor size, metastatic disease and EGFR) confirmed tumor dimension of 5–10cm ($p < 0.05$; $p = 0.02$), tumor dimension > 10 cm ($p < 0.01$; $p = 0.008$) and EGFR expression in range of 61% to 80% ($p < 0.05$; $p = 0.01$) as independent predictors of survival in patients with HCC.

DISCUSSION

About 50% of patients who have undergone curative treatment develop recurrence within 18 months and more than 60% of patients with HCC present in advanced stage, which in vast majority continue with the disease progression and poor outcome [8].

Liver transplantation is not available for patients of Republic of North Macedonia except for small number of patients who meet the criteria for Health insurance fund for a treatment abroad. Surgical therapy according the Barcelona-Clinic Liver Cancer (BCLC) staging system is appropriate for patients in stage 0 or very early HCC, when surgical resection is considered as a safe procedure [2, 14–16]. However, in surgical practice, resection of the liver also applied for tumors larger than those proposed by the BCLC classification system, sometimes tumors larger even more than 10 centimeters [16].

Surgical resection of the liver is contraindicated in cases with extrahepatic metastases, multiple and bilateral tumors, involvement of a large biliary duct, and with portal vein and vena cava tumor thrombosis [16]. Some authors have reported that liver resection for tumors larger than 10 cm, but without venous thrombosis, can be safely performed and that in a selected cases resection can be made after vascular thrombi removal [16, 17]. Surgical resection of large HCC, elective or urgent, in Republic of North Macedonia has been performed for many years, so in this study, we have included cases larger than 10 cm (7%–22.58% cases). Transarterial chemo-embolization and targeted therapies are also available for the patients in the last few years. The opportunity for target therapies for the patients with HCC and the new technology for DNA sequencing enabled us to approach this kind of studies.

In this study, we did not find mutations in EGFR exons 18–21 in patients with HCC as reported by Su MC et al in their study [11]. We analyzed the EGFR immunoexpression in HCC tissue samples and overexpression was found in the vast majority of cases. Nine (29.03%) patients had < 20% positive cells in the HCC tissue sample. The survival rate analysis showed that patients with 61%–80% EGFR positive cells present in the tissue sample had significantly shorter survival rate in comparison to patients with < 20% positive cells, but it was not a case in patients with 81%–100% EGFR positive cells. EGFR overexpression is reported to occur in 40% to 70% of conventional HCC and is associated with proliferative activity, stage, recurrence, more aggressive tumors, metastases, and a worse prognosis. EGFR overexpression is also detected in cirrhotic liver tissue [9, 11]. EGFR system of interaction between different signaling pathways plays an important role in HCC carcinogenesis from the early stages of disease. It also plays a role in liver reparation due to chronic liver damage. EGFR crosstalk system acts as a signaling hub for growth factors, cytokines and inflammatory mediators [18, 19]. Twenty-seven (87.09%) of our patients had underlying cirrhosis. In our study 61%–80% EGFR overexpression was correlated significantly to patient's survival, which we consider as a carcinogenic effect of EGFR. EGFR expression of 81%–100% which was not correlated to worse survival may have been due to hepatitis virus infection, inflammatory and reparatory process in the liver, as some authors have been reported [18, 19].

EGFR mutations play an important role in tumorigenesis of various malignancies and detecting EGFR mutations are reported to be a potential opportunity for therapy in patients with cancers [11]. Accumulation of multiple genetic

alterations leads to the development of HCC and identification of cancer type-specific oncogenes would provide new strategies for the development of molecularly targeted therapies [20]. Taking in consideration that we did not find the specific mutations in EGFR exons 18–21, further research on discovering other possible EGFR mutations and influence of other genes is needed in Macedonian population.

In conclusion, in this study, we confirmed EGFR overexpression in range of 61% to 80% as independent predictors of survival in patients with HCC, which means that EGFR inhibition could possibly improve survival rate of these patients. EGFR targeted therapies, monoclonal antibodies and small molecule tyrosine kinase inhibitors have given some benefits in the treatment of patients with HCC [3, 21], and research on EGFR mutations in different regions of the world might contribute to better understanding of the carcinogenesis and treatment outcome in HCC patients.

CONCLUSION

EGFR overexpression in range of 61% to 80% was an independent survival predictor in patients with HCC, implying that these patients could benefit from EGFR inhibition. However, the absence of EGFR mutations in exons 18–21 in any of the cases of this study suggest that single drug EGFR targeted therapy in patients with HCC may be insufficient. Further research and subclassification of these patients is needed in order to determine the most appropriate and beneficial therapy.

Introducere: *Terapiile țintite EGFR s-au dovedit benefice la pacienții cu carcinom hepatocelular (HCC). Totuși este nevoie de studii adiționale asupra populațiilor unde încă nu au fost evaluate expresia și profilul de mutații. Scopul studiului a fost de a evalua expresia EGFR la pacienții din Macedonia de Nord cu HCC fără mutația exonilor 18–21.*

Metode: *31 de cazuri cu HCC au fost incluse. Au fost evaluate prezența infecției HCV sau HBV, a cirozei, dimensiunea tumorală, prezența ganglionilor limfatici, nivelul alfa fetoproteinei precum și supraviețuirea. Prezența semnalului EGFR, precum și numărul ceulelor pozitive au fost luate în considerare.*

Rezultate: *Hepatita B și C, dimensiunea tumorală, boala metastatică și expresia EGFR au influențat supraviețuirea pacienților. Nu s-au observat mutații ale exonilor 18–21 la pacienții cu HCC. Expresia EGFR 61–80% tumorală a influențat semnificativ supraviețuirea ($p<0.01$). Modelul de regresie Cox a arătat că dimensiunea tumorii și prezența expresiei EGFR în proporție de 61–80% sunt predictori independenți pentru supraviețuire.*

Concluzii: *Expresia EGFR în proporție de 61–80% este predictor independent pentru supraviețuire. Acești pacienți ar fi cei ce ar beneficia cel mai mult de terapia cu inhibitori EGFR.*

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Conflict of interest disclosure: The authors declare no conflict of interests.

Author contributions: Dafina Nikolova collected data, performed the literature review, made the conception and design and drafted the original manuscript. Emilija Nikolovska Trpcevska contributed in collecting the data. Meri Trajkovska was responsible for critical revision and editing of the manuscript. Aleksandar Eftimov, Rubens Jovnovik and Vesna Janevska performed the molecular and genetic research. Vesna Janevska coordinated the study. All the authors approved the manuscript.

Institutional review board statement: The use of human tissue samples and clinical data were approved by the ethics committee of the Faculty of Medicine in Skopje (Republic of North Macedonia), and the research was carried out in accordance with the 1964 Helsinki Declaration.

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