TP53 Mutation in Correlation to Immunohistochemical Expression of P53 Protein in Patients with Hepatocellular Carcinoma

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Abstract

BACKGROUND: Mutations causing p53 inactivation are among the most common genetic alterations in human malignant tumours including hepatocellular carcinoma. Detection of p53 gene mutations in patients with hepatocellular carcinoma (HCC) should provide relevant data for the patients from the Republic of Macedonia and should allow the survivals additional therapeutic option as is gene therapy.

AIM: We aimed to detect p53 gene mutations in HCC tissue, and to correlate them with the immunoexpression of p53 protein and multiple clinicopathologic characteristics of a tumour.

MATERIAL AND METHODS: We analysed thirty patients with HCC for multiple clinic-pathological characteristics. Tumour tissue samples were immunostained for p53 and detection of p53 gene mutations was performed by polymerase chain reaction followed by Sanger sequencing.

RESULTS: Changes in p53 gene sequence were detected in four patients (13.33%), one of them a polymorphism and the other three were missense point mutations with p53 immunoexpression of 50%, 0%, 0% and 90%, respectively. All patients with p53 mutations had cirrhosis. Two of them had Hepatitis B infection, moderately differentiated tumour and T2 status. There was one case with a well-differentiated tumour and one with T4 status. All of them were with vascular invasion. The size of the tumours was in the range of 2.5 cm to 16 cm. All 3 mutations were located in exon 7.

CONCLUSION: Mutations in p53 gene are not always associated with obviously altered immunoexpression of p53 protein. Detection of p53 gene mutations is necessary in each case because the new therapeutic modalities offer to apply gene therapy.

Introduction

Tumour suppressor gene p53 plays a key role in cell cycle regulation, cell proliferation and apoptosis following DNA damage. p53 is responsible for maintenance of genomic integrity [1] [2]. Inactivation mutation of p53 is among the most common genetic alterations in human cancers, including hepatocellular carcinoma (HCC). The mutational spectrum of p53 gene differs in HCC from different geographic regions. This difference comes due to differences in population exposition to Aflatoxin B1 and Hepatitis B infection (HBV). Transversion of guanine to thymine at the third position of codon 249 is a common finding in patients with HCC from southern Africa and southern China [2].

In contrast, in regions where exposure to Aflatoxin B1 is low, there are almost no mutations in codon 249, or they occur less significantly [2] [3] [4] [5] [6] [7]. Population in the Republic of Macedonia is exposed to Hepatitis B infection with the incidence of 7.8 in the 2013 year [8], and the same year Aflatoxin M1and B1 contamination is found in raw milk and feed [9]. In the neighbouring countries, the contamination of raw milk with Aflatoxin Ma and B1 was detected

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earlier [10]. According to EUROCAN the incidence rate of liver cancer and intrahepatic bile duct cancer in the Republic of Macedonia is 8.4, and the mortality rate is 11.1 [11].

Mutations of p53 gene or positive immunostaining for mutated p53 protein can be used as a significant indicator of poor prognosis in patients with HCC [12] [13]. Modern therapeutic possibilities such as surgical resection, liver transplantation, percutaneous ablation and transcatheter chemoembolization provide a better prognosis for patients, but the overall survival rate in patients with HCC remains low [11]. Hence, great importance for patients with HCC is the development of multidisciplinary interventions and new therapeutic approaches, including biotherapy. The efficacy of chemo and radiotherapy may be elevated by an endogenous or exogenous normal type of p53. There are currently several in vitro and in vivo studies with products for modulating the p53 status [14].

Advexin (for adenoviral p53), ONYX-015, CNHK200, SCH58500 are products in clinical development, while Gendicine-a gene therapy product mainly built from normal p53 and modified adenovirus is already approved for commercial use by the Chinese State Food and Drugs Administration [15] [16] [17] [18] [19]. Due to the new therapeutic possibilities, and because the presence of the mutant p53 gene in HCC is an indicator of a poor prognosis, the detection of the mutant gene and the presence of its protein are extremely important for patients with HCC.

This study aims to determine the mutations of the p53 gene and correlate them with the immunohistochemical expression of its product, as well as to correlate them with the clinical and pathological characteristics of a tumour in 30 cases of HCC in patients from the Republic of Macedonia.

Material and Methods

We analysed 30 patients with histologically proven HCC, diagnosed and treated at the University Clinic of Gastroenterology and Hepatology and the University Clinic of Abdominal Surgery in Skopje

Following parameters were determined by echosonography and computed tomography images: the dimension of tumour node, the multiplicity of the tumour nodes, the presence of tumour emboli in the large vessels and the presence of cirrhosis in the surrounding liver tissue. Serological tests for hepatitis B and C infection were performed in all patients. All patients were followed up until the death.

During the diagnostic procedure of HCC at the Institute of Pathology in Skopje, the grade of

differentiation, vascular invasion, T category of pTNM classification (AJCC 2017) and the presence of cirrhosis in the surrounding, peritumoral liver tissue was determined. Additionally, immunohistochemical staining for p53 and PCR for the detection of p53 gene mutations were performed.

Immunohistochemical stainings with an antibody against p53 (Monoclonal Mouse, Anti-Human, Clone DO-7, DACO, dilution 1:50) using Avidin-Biotin immunoperoxidase technique were made. For the visualisation of the antigen-antibody reaction, LSAB and En-Vision kit from DAKO was used. The results of immunostaining were determined as a percentage of positive nuclear signals for p53.

For the determination of p53 gene mutation polymerase chain reaction (PCR) with subsequent Sanger sequencing of exons 2-11 of the p53 gene were performed. The primers for each exon are shown in Table 1.

Table 1: Nucleotide sequences of primers used for amplification (p53 priming sequences)

Oligonucleotide	Oligo sequence (5' to 3')	Product size
p53Ex2-3F	tctcatgctggatccccact	365
P53Ex2-3R	agtcagaggaccaggtcctc	
p53Ex4F	tgaggacctggtcctctgac	413
p53Ex4R	agaggaatcccaaagttcca	
p53Ex5-6F	tgttcacttgtgccctgact	467
P53Ex5-6R	ataacccctcctcccagaga	
p53Ex7F	cttgccacaggtctccccaa	263
P53Ex7R	aggggtcagaggcaagcaga	
p53Ex8-9F	ttgggagtagatggagcct	445
P53Ex8-9R	agtgttagactggaaacttt	
p53Ex10F	caattgtaacttgaaccatc	260
P53Ex10R	ggatgagaatggaatcctat	
p53Ex11F	agaccctctcactcatgtga	245
P53Ex11R	tgacgcacacctattgcaag	

DNA was extracted from deparaffinized tissue sections in polypropylene tubes using Dynabeads DNA kit (Invitrogen, ThermoFisher Scientific).

Evaluation of the quality and quantity of the isolated material was made using horizontal agarose gel electrophoresis and spectrophotometer (Thermo Scientific Evolution 260 Bio spectrophotometer).

Polymerase Chain Reaction (PCR)

For amplification of the DNA regions of interest, standard polymerase chain reaction was performed. The PCR reaction was performed on AutoQ server thermal cycler (Quanta Biotech).

Detection of TP53 gene mutations

Detection of TP53 gene mutations in the amplified exons was carried out with Sanger DNA sequencing on automated genetic analyser ABI 310 Genetic Analyzer (Applied Biosystems®).

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Sequencing of the amplified DNA fragment

The technique of DNA sequencing was performed using the commercial Big Dye Terminator v.1.1 Cycle Sequencing Kit. Two µl of the PCR product were used to perform the sequencing reaction, 2 µl of 2.5 X Big Dye Terminator v.1.1 Cycle Sequencing, 1 µl 5 X Big Dye Sequencing Buffer and 1 µl of 10 pmol forward or reverse primer in a total volume of 10 µl.

After the sequencing reactions competed, capillary gel electrophoresis of the purified mixture was performed on automated genetic analyser ABI 310 under the conditions of the BDx_Standard_Seq_Assay_POP4 module. The electrophoresis products were subsequently analysed with the protocol KB_310_POP4_BDTv1.1 through the software Sequencing Analysis v5.4 and TP53 gene mutations were confirmed through the SeqScape v2.7 software.

Results

Ten patients out of 30 (33.33%) were female, and 20 (66.66%) were male, aged 38 to 76, with a mean age of 59.13 years. Twenty-three patients (76.66%) were serologically positive for hepatitis B, two patients (6.6%) were seropositive for hepatitis C, and 5 (16.66%) patients were seronegative to both B and C hepatitis. Liver cirrhosis was detected in 28 (93.33%) patients. In the most of the patients the local growth was determined as T2 - 13 cases (43.33%), in 5 (16.66%) patients the local growth was determined as T1, in 10 (33.33%) patients it was T3 and one patient (3.33%) had T4 status of the local growth.

Vascular invasion was detected in 20 (66.66%) patients. Six patients (20%) had multiple HCC nodes in the liver, and the remainder 10 patients (33.33%) had solitary tumours. The smallest tumour node measured 3.5 cm and largest 16 cm. Clinicopathological characteristics of the analysed group of patients and immunoexpression of p53 in HCC tissue are shown in Table 2.

Immunohistochemical staining with the antibody against p53 showed positivity in the range from 0% to 90% in the cell nuclei (Figure1). Changes in p53 gene sequence were detected in four patients (13.33%), but one of them a polymorphism, and the other three were missense point mutations. So, p53 gene mutation was found in 3 (10%) out of 30 patients, highlighted in Table 2, under the numbers 10, 20, and 21.

All 3 mutations were heterozygous point mutations, in exon 7. No mutations were found in codon 249. The polymorphism in codon 247 (exon 7) was detected in patient 7. Missense point mutations in codon 260 (exon 7) was detected in patient 10, in codon 245 (exon 7) in patient 20, and in codon 242 (exon 7) in patient 21 (Figure 2).

Two patients with missense mutations were male, and one was female at the age of 38 to 75 years, mean 60.66 years. Two of the patients had hepatitis B infection, and one was negative. All patients had cirrhosis and solitary tumours ranging in size from 2.5 cm to 16 cm. All showed the presence of vascular invasion. Two of the tumours were moderately differentiated; one was well differentiated. Two of them showed a T2 tumour local growth, and one was T4 tumour status. The percentage of the p53 immunohistochemical expression ranged from 0 to 90 per cent.

Table 2: Clinicopathological characteristics of the patients and p53 immunoexpression

Patient	Gender	Age	Hepatitis B C		Cirrhosis	Tumour node	Tumour size**** Cm	Vascular invasion	G	T	P53 %	Survival Months
1	f*	42	+	-	+	M***	12	-	2	3	5	8
2	m**	63	+	-	+	1	7	+	2	2	20	1
3	m	66	+	-	+	1	11	+	2	2	25	26
4	m	57	+	-	+	M	9	+	2	3	30	21
5	f	59	+	-	+	1	3.5	+	2	2	50	23
6	m	76	+	-	+	M	3.5	-	1	2	20	70
7	m	65	+	-	+	1	3.5	+	2	2	50	13
8	m	66	+	-	+	1	3.8	-	2	1	50	24
9	f	75	-	+	+	M	12	+	3	2	30	1
10	f	38	+	-	+	1	10	+	2	4	90	12
11	f	73	+	-	+	1	2.5	+	2	1	85	62
12	m	53	+	-	+	1	9	+	2	2	80	1
13	m	52	+	-	+	1	9	-	3	1	30	17
14	m	56	+	-	+	1	3	-	1	1	25	13
15	m	74	1	-	+	1	10	+	1	1	50	13
16	f	59	+	-	-	1	15	+	2	3	20	9
17	f	71	-	-	+	1	10	+	1	3	80	9
18	f	57	+	-	+	M	10	+	2	4	70	3
19	m	67	+	-	+	M	6	-	1	2	90	25
20	m	75	+	-	+	1	2.5	+	1	2	0	8
21	m	69	-	-	+	1	16	+	2	2	0	4.5
22	m	42	+	-	+	1	12	-	2	3	90	24
23	m	61	+	-	+	1	10	+	1	3	80	7
24	m	50	-	+	+	1	11	+	2	3	90	6
25	m	48	+	-	+	1	14	-	1	2	70	6
26	f	67	-	-	+	1	12	-	1	2	40	11
27	f	48	+	-	+	1	10	+	3	3	1	7
28	m	65	-	-	+	1	7	+	1	3	20	8
29	m	52	+	-	-	1	6.5	-	1	3	10	23
30	м	58	-	-	+	1	3	+	2	2	80	2

* Female, ** Male, ***Multiple tumour nodes in the liver. **** If multiple nodes present - the greatest dimension of the greatest node is shown in the table.
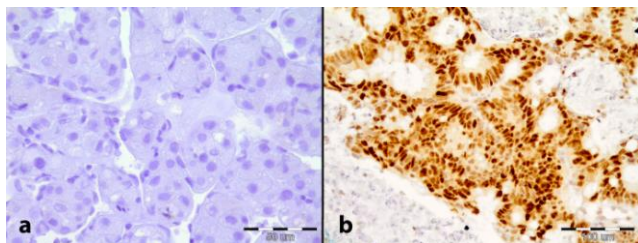
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Figure 1: Micro-image of immunohistochemical staining for p53 in HCC tissue. a) Negative immunostaining for p53 (patient No. 20) (10 x 40) b). Variable intensity of immunostaining in about 90% of the tumour tissue in this microscopic field. The surrounding non-coloured tissue represents a cirrhotic tissue in which a tumour infiltrates (patient No. 10), (10 x 20)

In tumour samples where p53 missense mutations in codons 245 and 242 were detected, the immunoexpression of p53 was 0% (cases 20 and 21), and the tumour sample with a p53 missense mutation in codon 260 (case 10) showed an immunoexpression of p53 in approximately 90% of the tumour cells.
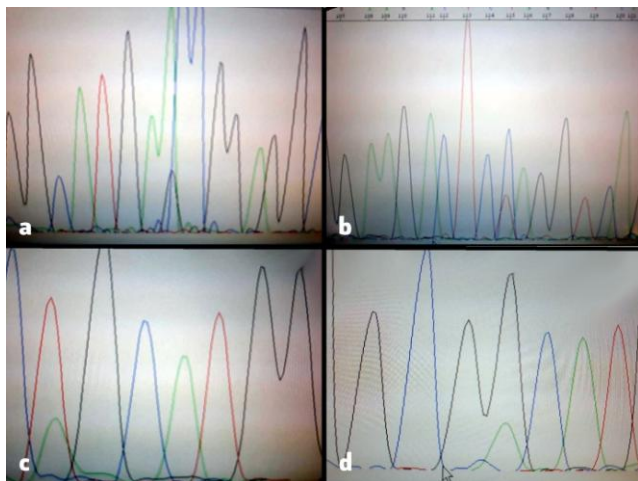

Figure 2: a) AAC> AGC LYS247SER (Patient 10) b) Polymorphism TCC> TCT SER260SER (patient7) c) GGC> GAC GLY245ASP (Patient 20) d) TGC> AGC CYS242SER (Patient 21)

The survival time of the entire group was 1 to 70 months, mean survival 15.25 months. Patients with missense mutations in p53 gene had a mean survival of 8.16 months (min. 4.5; max. 12), while those without p53 mutation had a mean survival time of 16 months (min. 1; max. 70; SD = 16.58). However, intergroup differences were insignificant (P > 0.05), probably due to the small number of cases in the group with mutated p53.

Discussion

The p53 gene is located on the 17th chromosome (17p13.1) and encodes phosphoprotein,

formed by 393 amino acids. It consists of 4 units (domains): a) a domain that activates transcription factors, b) a domain that recognises specific DNA sequences, c) a domain responsible for tetramerisation of the protein, and d) a domain that recognises damaged DNA. The protein responds to different cellular stresses to regulate the expression of target genes, thereby induces cell cycle arrest, apoptosis, ageing, DNA repair, or changes in metabolism [20] [21].

The level of p53 protein in normal cells is low, and the wild type of p53 is a labile protein. Cellular stress can trigger a rise in the p53 protein to fulfil its function as a "genome keeper" [20] [21] [22].

P53 gene mutations are the most commonly reported somatic mutations in human neoplasms. Reports have been published in which mutations in the p53 gene, or positive immunostaining for p53, are associated with a higher grade of neoplasms and a more advanced stage in various types of malignant tumours. In many reports p53 mutations are considered a strong marker that predicts an increased risk of local relapse, failure of therapy and poor survival in many types of human neoplasms, such as breast cancer [24], colorectal cancer [25], cancer of esophagus [26], lung [27], ovarian cancer [28] and head and neck cancer [29].

Several studies suggest that p53 mutations are involved in determining the differentiation, proliferative activity, and progression of the HCC. p53 mutations are also associated with marked HCC invasiveness and may influence the postoperative course and occurrence of relapses [20] [21] [22] [23]. Additionally, p53 mutations or overexpression of the mutant p53 protein can be used as a significant indicator of a poor prognosis [30] [31] [32] [33] [34] [35] [36]. However, for the predictive evaluation of HCC, the p53 effect should be considered in correlation with other significant factors such as tumour size, Child-Pugh score, TNM stage and vascular invasion [14].

Positive immunoexpression of the mutant p53 protein is detected in 37% HCC, but the overexpression of the p53 protein does not always depend on the p53 mutation [37] [38] [39].

Hence, mutations in the p53 gene in patients with HCC, in each case are of particular importance, especially for the development of new modalities of therapy and the application of gene therapy. Today's therapy for patients with HCC is with limited efficacy. The development of gene therapy for HCC, such as the use of apoptotic genes, genes that code anti-angiogenic factors or immunomodulatory molecules, gives hope for a longer survival of patients with HCC [15] [16] [17] [18].

In this study, three mutations of the p53 gene were found in three different patients out of 30

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analysed. In two of the tumour samples with a p53 missense mutation, we did not find any immunoexpression of the p53 protein, although the mutation was present. In the third case, the immunoexpression of the pathological p53 was 90% of cells.

The survival time of patients with confirmed mutations in this study was shorter than the survival time of the remaining patients, however statistically insignificant (P > 0.05). So, the detection of the mutant p53 gene is necessary for each patient with HCC, to build a strategy for application of appropriate therapy.

Detection of mutations in the p53 gene in survivors will also provide a gene therapy option with p53 products already in commercial use or in development [15] [16] [17] [18].

Compliance with Ethical Standards

All procedures performed in studies involving human participants were by the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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