UDC 575
https://doi.org/10.2298/GENSR1702399A
Original scientific paper

STK11 GENE MUTATIONS AMONG PATIENTS WITH SPORADIC BREAST CANCER

Georgi ANTOV[1], Maria KRASTEVA[1], Silvia ANDONOVA[2], Alexey SAVOV[2], Svetla ANGELOVA[1], Lubomir STOILOV[1], Draga TONCHEVA[3]

[1]Laboratory of Genome dynamics and stability, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria,
[2]National Genetic Laboratory, UHOG "Maichin dom", Medical University Sofia, Sofia, Bulgaria,
[3]Department of Medical Genetics, Faculty of Medicine, Medical University Sofia, Sofia, Bulgaria

Antov G., M. Krasteva, S. Andonova, A. Savov, S. Angelova, L. Stoilov, D.Toncheva (2017): *STK11 gene mutations among patients with sporadic breast cancer.*- Genetika, Vol 49, No. 2, 399 - 413.

Germline mutations affecting *STK11* (LRG_319) are profoundly studied in relation to Peutz-Jeghers syndrome, predisposing to the development of various cancers at multiple sites. Though somatic mutations in *STK11* are found to be present in several cancers, limited data on its involvement in sporadic breast cancer are available. The present study aims to evaluate the frequency and spectrum of genetic alterations in *STK11* in a group of Bulgarian patients with sporadic breast cancer. A total of 73 tumor and 22 corresponding blood specimens derived from the patients, and 10 blood samples from clinically healthy controls were analyzed. High Resolution Melting analysis followed by Sanger sequencing and bioinformatic prediction tools were utilized. Seven patients (9.58%) harbored *STK11* alterations, only two (2.74%) of which are exonic: one nonsense c.322A>T; p.K108X (deleterious) and one missense c.440G>A; p.Arg147His (of unknown significance). Two intronic variants were also observed: c.290+36G>T and c.*16+18C>A (novel). To our knowledge the results represent the first data indicating presence of *STK11* alterations in patients with sporadic breast cancer. The limited number of the detected deleterious mutations indicates that mutational inactivation of the gene is a rare event and probably plays a minor role in sporadic breast carcinogenesis.

Keywords: High Resolution Melting Analysis, Mutation, Sporadic breast cancer, *STK11*, Tumor suppressor gene

INTRODUCTION

Breast cancer (BC) is the most common malignant disease and a leading cause for cancer death among women worldwide. In 2012, 1.67 million new cancer cases have been diagnosed (25% of all cancer cases) and 522,000 deaths established worldwide (FERLAY *et al.*, 2012). In Bulgaria, the newly registered cases for 2013 are 3997, of which 1274 deaths have been reported

Corresponding author: Georgi Georgiev Antov, Laboratory of Genome dynamics and stability, Institute of Plant physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Bldg. 21, 1113, Sofia, Bulgaria, Tel.: ++359 2 9792617, Fax: ++359 2 8739952, Mobile: ++359 895020134, E-mail: antov8107@abv.bg

(VALERIANOVA *et al*., 2015). BC is a very heterogeneous disease and exhibits various histopathological and biological characteristics, distinct clinical outcome and diverse treatment responses. Genetic and/or epigenetic alterations affecting tumor suppressor genes and proto-oncogenes, as well as other factors such as age, race/ethnicity, lifestyle and environment are implicated in breast cancer development and progression.

The tumor suppressor gene *STK11* (serine/threonine kinase 11, OMIM* 602216), also known as *LKB1* (19p13.3) (HEMMINKI *et al*., 1997), extends over 23 kb of genomic DNA. It is composed of nine coding and one non-coding exons situated in the 3′ untranslated gene region and transcribed in telomere-centromere direction. Encoded 48.6 kDa protein covers 433 amino acid (aa) residues and represents a multitasking master kinase involved in the maintenance of genome stability (SAPKOTA *et al*., 2002), in p53−dependent apoptotic pathway (KARUMAN *et al*., 2001) and cell survival (SHAW *et al*., 2004). Furthermore, it is shown to be involved in the control of cell metabolism (WOODS *et al*., 2003), in the regulation of the cellular growth (CORRADETTI *et al*., 2004) and in the inhibition of cancer invasion and metastasis (ZHUANG *et al*., 2006).

The first data demonstrating involvement of *STK11* in cancer diseases is the work of HEMMINKI *et al*. (1998) and JENNE *et al*. (1998) who established that germline pathogenic mutations in the gene are predisposing to the development of Peutz−Jeghers syndrome (PJS, OMIM #175200). The syndrome represents a rare cancer-prone, autosomal−dominantly inherited disease (PEUTZ, 1921; JEGHERS *et al*., 1949), which is characterized with the formation of hamartomous polyps in the gastro-intestinal tract, melanin spots on the lips and buccal mucosa. PJS patients are highly predisposed to the development of various cancers at multiple sites, such as gastrointestinal tumors (38 − 93%), breast (32 − 54%), pancreatic (11 − 36%), gynecological (13 − 18%) and lung carcinomas (up to 15%) (GIARDIELLO *et al*., 1987; GIARDIELLO *et al*., 2000; SPIGELMAN *et al*., 1989). Germline pathogenic mutations in *STK11* in patients with PJS have been identified with a frequency of 30% to 80% depending on the studied population and the applied screening techniques (HEARLE *et al*., 2006).

Additionally, a number of studies indicate presence of somatic mutations in *STK11* in sporadic cancers, such as testicular tumors (AVIZIENYTE *et al*., 1998), colorectal cancer (DONG *et al*., 1998), pancreatic carcinoma (SU *et al*., 1999), melanoma (ROWAN *et al*., 1999), lung adenocarcinoma (SANCHEZ-CESPEDES *et al*., 2002).

A limited number of studies explored the significance of *STK11* for sporadic breast carcinogenesis. Though no pathogenic *STK11* mutations were established, allele loss of the locus was relatively frequently observed in sporadic BC disease amounting to 8% (3/40) (BIGNELL *et al*., 1998) and 17% (5/30) (FORSTER *et al*., 2000). Furthermore, a few investigations have demonstrated a relation between the low expression of STK11 and clinico-pathological features of patients with sporadic BC with respect to higher histological grade, larger tumor size, presence of lymph node metastasis and poor survival (SHEN *et al*., 2002; FENTON *et al*., 2006).

Still, detailed data regarding the involvement of *STK11* in sporadic breast cancer globally are incomplete. In this context, the present study aims to evaluate the frequency and spectrum of genetic alterations in *STK11* gene in a group of Bulgarian patients diagnosed with sporadic BC through application of High Resolution Melting (HRM) assay followed by Sanger sequencing.

PATIENTS AND METHODS

The group under study included 73 Bulgarian female patients with sporadic primary invasive breast carcinoma treated at the Bulgarian National Oncological Centre Hospital, Sofia,

between 2000 and 2003. Cases were staged according to the TNM classification of Union International Contre le Cancer (UICC) and followed up for a 5-year period. The age ranged from 29 to 78 years. The patients were previously characterized according to several molecular-genetic characteristics including abnormal status of *p53*, *BRCA1*, *ATM*, *PIK3CA, CHEK2* and *HER2* genes (BOZHANOV *et al*., 2010; ANGELOVA *et al*., 2012; KRASTEVA *et al*., 2012; KRASTEVA *et al*., 2014).

Table 1. Patients' clinico-pathological and tumor characteristics.

Variable		n = 73
Age (A)	Years (mean)	53,2
	Range	29 − 78
	< 50	30
	>/= 50	43
Tumor size (T)	T1	35
	T2	30
	T3	5
	T4	3
Nodal status (N)	N0	45
	N+	28
Grade (G)	G1	3
	G2	59
	G3	11
Histologic type (H)	Lobular	8
	Ductal	65
Estrogen receptor (ER) status	ER+	38
	ER-	35
Progesterone receptor (PR) status	PR+	41
	PR-	32
HER2 overexpression	HER2+	8
	HER2-	33
	Unknown	32
p53 mutation*	*p53+*	14
	p53-	59
ATM mutation*	*ATM+*	10
	ATM-	63
PIK3CA mutation*	*PIK3CA+*	21
	PIK3CA-	52
CHEK2 mutation*	*CHEK2+*	5
	CHEK2-	68
BRCA1 hypermethylation*	*BRCA1+*	10
	BRCA1-	61
	Unknown	2

n − number of patients * Data obtained from previously published studies (BOZHANOV *et al*., 2010; ANGELOVA *et al*., 2012; KRASTEVA *et al*., 2012; KRASTEVA *et al*., 2014)

Based on these data summary of the patients' clinico-pathological and tumor features is presented on Table 1. Corresponding blood DNA specimens from 22/73 patients were also analyzed. The control group consisted of 10 clinically healthy persons without family history of breast cancer.

All participants gave an informed consent for conducting the research. Clinical information was obtained from the existing medical records and is presented in a way preventing patients' identification.

DNA isolation

Tumor DNA was isolated from fresh frozen breast tumor specimens by a standard Proteinase K/Phenol procedure including tissue homogenization in lysis buffer at 37°C for 48 h followed by phenol/chloroform/isoamyl alcohol purification and ethanol precipitation. DNA samples were obtained from peripheral blood of the patients and healthy controls according to the modified standard Proteinase K/Phenol procedure (AHMAD *et al*., 1995). DNA concentration and quality was determined using BioSpec-nano Spectrophotometer (Shimadzu Biotech, Japan).

High Resolution Melting analysis (HRM)

The 9 coding exons of *STK11* were analyzed using a total of 12 primer pairs, 7 of which were chosen according to literature data (DONG *et al*., 1998; BIGNELL *et al*., 1998; CONNOLLY *et al*., 2000) and 5 were designed by the use of AnnHyb software (http://bioinformatics.org/annhyb/). Primer sequences, size of the amplicons and optimal annealing temperatures (Ta, determined experimentally) are listed on Table 2.

HRM analysis was performed using 5 × HOT FIREPol® EvaGreen® HRM Mix - no ROX (Solis BioDyne, Estonia). Each reaction was carried out in a final volume of 5 µl containing 20 ng of gDNA, 0.1 µl of 10 pmol/µl primers (forward and reverse), 1 × HRM Mix and ddH_2O to the final volume. The PCR cycling parameters were as follows: denaturation at 95°C for 15 minutes, followed by 40 cycles of pre-incubation at 95°C for 0.25 seconds, annealing for 0.25 seconds at the appropriate annealing temperature and extension at 72°C for 0.25 seconds.

The HRM step was performed on LightCycler®480 (F. Hoffmann-La Roche Ltd., Switzerland) and PikoReal™ Real-Time PCR System (Thermo Fisher Scientific, Inc., USA) systems and was carried out over the range from 60°C to 99.9°C raising by 0.2°C/0.2 seconds at each step.

Sanger sequencing analysis

All amplified PCR products with altered HRM profile were analyzed by Sanger sequencing. An aliquot of 1.5 µl of each product was subjected to Exonuclease – Shrimp Alkaline Phosphatase clean-up enzymatic digestion by ExoSAP−IT (Affymetrix, USA) according to the manufacturer's instructions. Sequence reactions were carried out on the ExoSAP-IT-purified PCR products using BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, USA). The reaction was carried out in a final volume of 10 µl, containing 2.5 µl of the product, 0.4 µl BigDye Terminator Mix, 0.4 µl of 10 pmol primer (forward or reverse), 2 µl of 5 × Sequencing Buffer and dH_2O to the final volume. After an initial denaturation step at 96°C for 5 minutes, 30 cycles of the reaction were performed at: 96°C for 20 seconds, 55°C for 20 seconds and 60°C for 2 minutes, followed by a final extension step at 60°C for 5 minutes. Samples were dissolved in HiDi Formamide after EDTA/NaOAc/Ethanol precipitation and processed on an ABI Prism 3130

Genetic Analyzer (Applied Biosystems, USA). The results were analyzed using Chromas Lite 2.1.1 software (Technelysium Pty Ltd, Australia).

Table 2. Primers used for HRM analysis of the STK11 gene

STK11 exon	Primers	5′ → 3′	Product length, bp	Ta, °C
1U	F	GGAAGTCGGAACACAAGGAA	240	66
	R	CAGGTCCCCCATCAGGTACT		
1D	F	TTCATCCACCGCATCGACTC	280	67
	R	GACAGAACCATCAGCACCGTGACTG		
2	F	GGCCCTTTCCCACAGCACT	271	66
	R	AGGCCCCGCGGTCCCAACA		
3	F	CCCCCGTGCTCCCTGGGCCTGT	173	70
	R	CCCTGCCCCGCGCACGCA		
4	F	CGGCCCCAGGACGGGTGT	218	69
	R	CTCAGGGAGTGCCCGGGAGG		
5	F	CCCTCCCGGGCACTCCCTGAG	254	71
	R	GCCGGCAGCTGCCCAAGACG		
6A	F	TCAACCACCTTGACTGACCA	251	66
	R	ACACCCCCAACCCTACATTT		
6B	F	GACCACGCCTTTCTTCCCTCCC	236	66
	R	ACACCCCCAACCCTACATTT		
7	F	CAGCTGACAGGCTCCTCGC	159	66
	R	CTCAACCAGCTGCCCACAT		
8	F	CCTGACAGGCGCCACTGCTTC	271	70
	R	CATCCTGGCCGAGTCAGCAGAGC		
9U	F	CTCAGGCCACACTTGCCG	137	64
	R	CCTGGATTTGGTGCTCAGCTGC		
9D	F	AAGGCCGTGTGTATGAACGG	206	67
	R	ATCCAGGCGTTGTCCCCAC		

***In silico* analysis**

In order to evaluate theoretically the possible functional consequences of the detected alterations in *STK11* five software predictor programs were applied: PROVEAN (http://provean.jcvi.org), SIFT (http://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), LoFtool (http://www.ensembl.info/ecode/loftool/) and RegulomeDB (http://regulomedb.org). The change in protein structure was analyzed by SWISS-MODEL software (http://swissmodel.expasy.org). For the splice-site analysis the web-based tools GeneSplicer (PERTEA *et al*., 2001), MaxEntScan (YEO and BURGE, 2004) and Human Splicing Finder v3.0 (DESMET *et al*., 2009) were used. Genomic variation frequencies are given according to the HapMap (http://hapmap.ncbi.nlm.nih.gov/index.html.en) and 1000 Genomes (http://www.1000genomes.org) projects.

RESULTS

At the first stage of our study, 73 tumor samples derived from patients with sporadic BC were subjected to conventional HRM analysis of the entire coding sequence of *STK11*. Samples with altered HRM profile were subjected to Sanger sequencing. USCS Genome Browser on Human (GRCh38/hg38) Assembly was used for sequence data interpretation. From all patients enrolled, seven have shown altered HRM profiles (9.58%). Overall, four types of alterations were identified: c.322A>T, c.440G>A, c.290+36G>T, c.*16+18C>A (Table 3). No correlations between *STK11* mutational status and the clinico-pathological characteristics of the patients were observed (data not shown). All patients with alteration in *STK11* survived the 5-year follow-up period.

Table 3. STK11 variants detected in patients with sporadic BC and in silico predictive results.

Patient №	Position	DNA change	Variant	PREDICTED EFFECT					
				PROVEAN	SIFT	PolyPhen-2	LoFtool	HSF 3.0	RegulomeDB
39	Exon 2	c.322A>T	p.K108X	Deleterious	–	–	–	–	–
72	Exon 3	c.440G>A	p.R147H	Deleterious	Deleterious	Benign	Probably damaging	–	–
7	Intron 1	c.290+36G>T	non-coding	–	–	–	Probably damaging	Potential splicing alteration	Likely to affect TF binding and linked to the expression of the gene target
6, 24, 49, 63	Intron 9	c.*16+18C>A	non-coding	–	–	–	–	Potential splicing alteration	–

Only two patients (2.74%) harbored exonic mutations: one nonsense c.322A>T, p.K108X (deleterious) and one missense c.440G>A, p.Arg147His (variant of unknown significance).

Neither of the exonic mutations was established in the control group. The c.322A>T substitution was not found in the DNA sample from peripheral blood of the same patient.

The nonsense mutation maps to exon 2 of the gene (Chr19:1218448) (Figure 1), leading to replacement of the Lys residue (AAA) with a Stop codon (TAA) at a position 108 in STK11 protein (p.K108X), causing premature protein truncation. The mutant p.K108X protein contains only N-terminal domain and part of the kinase domain, being significantly changed in comparison to the wild type as predicted by the Swiss-Model software (Figure 2 − A and B). A deleterious outcome of the mutation was suggested via PROVEAN (Protein Variation Effect Analyzer) (Table 3).

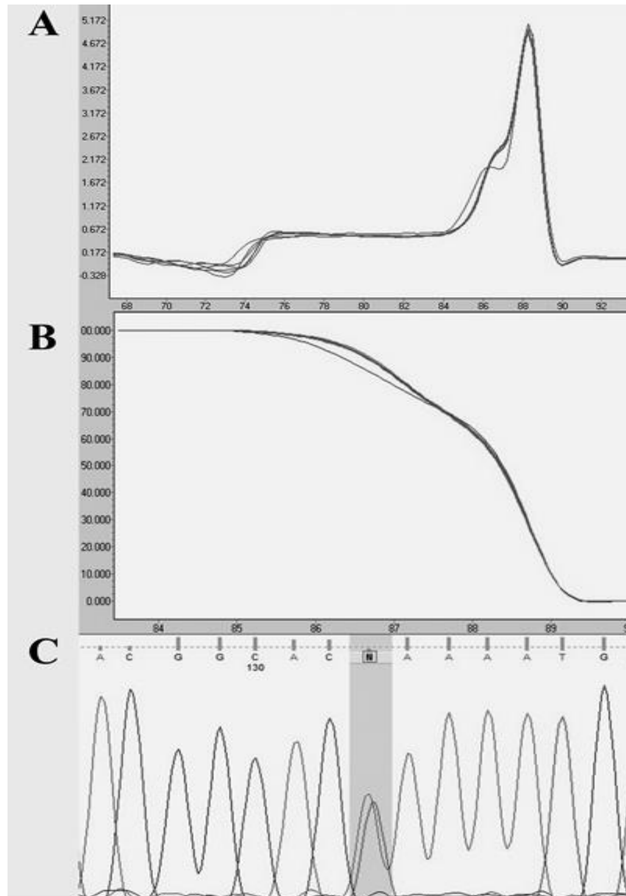

Figure 1. HRM and sequencing analysis data for c.322A>T in exon 2 of *STK11*: A − Melting peaks; B – Normalized and shifted melting curves; C – Sequencing profile.

The missense mutation c.440G>A was positioned at Chr19:1219389 in exon 3 of *STK11* (Figure 3), resulting in substitution of Arg with His at 147 position of the protein (p.Arg147His). Swiss-Model software was used to estimate the mutation effect on the protein structure. No significant change was predicted (sequence identity − 99.34%) compared to the wild type (Figure

2 − A and C). To further clarify the functional effect of the variant c.440G>A, the web-based PROVEAN, SIFT, LoFtool and PolyPhen-2 software were applied. SIFT and PROVEAN established deleterious effect, LoFtool defined it as probably damaging, while PolyPhen-2 classified it as benign (Table 3).

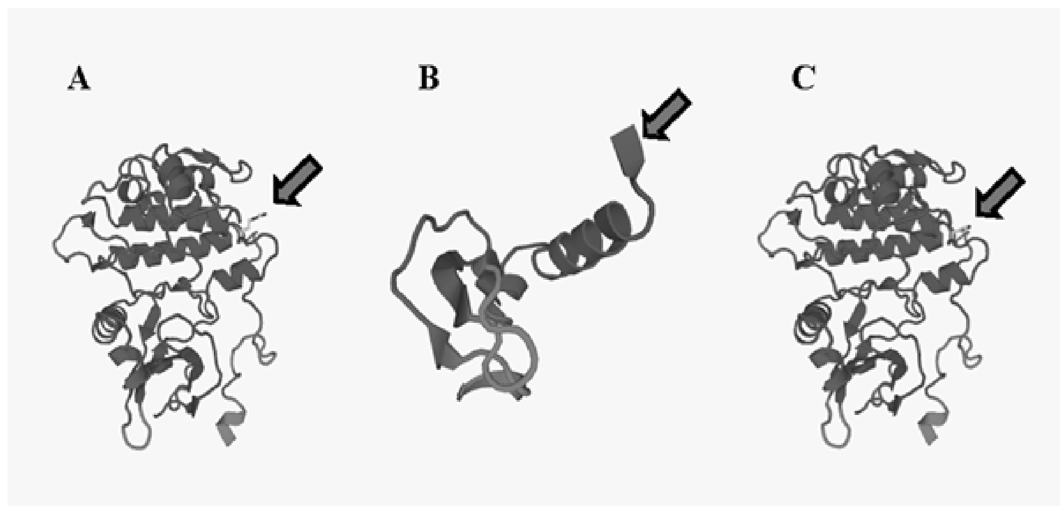

Figure 2. SWISS−MODEL predicted tertiary structure of the human STK11 protein; A − wild type; B − mutant type p.K108X; C − mutant type p.R147H.

Two of the established alterations were intronic variants: c.290+36G>T, c.*16+18C>A (g.1226681C>A). The variant c.290+36G>T was present in the tumor and the corresponding blood sample of one patient and absent in the control group. The change was situated at chr19:1207239 in intron 1 of the *STK11*. *In silico* evaluation obtained through the HSF web program predicted activation of an intronic cryptic donor site at the position where the additive intronic splicing enhancer (ISE) resides, which impedes the binding of the SR protein SC35 and leads to splice alteration of the *STK11*. In relation to this, the polymorphism was cross-checked on RegulomeDB database and LoFtool software. RegulomeDB predicted that the observed variant is likely to affect transcription factors binding and is linked to the expression of the gene target, while LoFtool predicted probably damaging effect (Table 3). The search for the functional outcome based on GeneSplicer and MaxEntScan software was non-informative.

The variant c.*16+18C>A (g.1226681C>A) was detected in four patients under study, in the corresponding blood sample of one of the patients, and in three clinically healthy persons as well. The intronic variant c.*16+18C>A represents a single base substitution C to A situated at the 5′ end of intron 9, 34 bases apart from the exon 9/intron 9 splice-site junction. Functional evaluation was performed by the application of Human Splicing Finder 3.0 software. Bioinformatic data predicted changes in the Cis–acting elements, namely the alteration of intronic splicing silencer (ISS) site and creation of new intron splicing enhancer (ISE) site, resulting in alteration of splicing (Table 3).

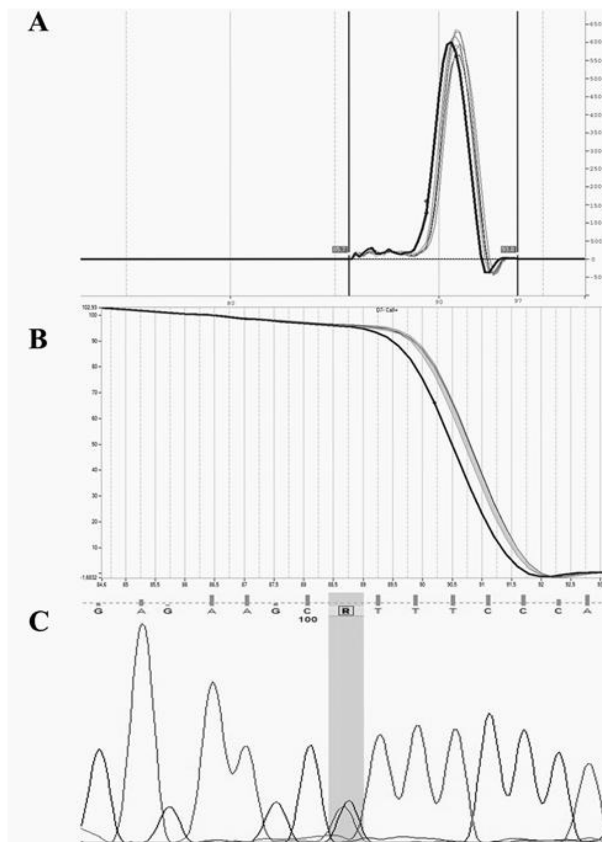

Figure 3. HRM and sequencing analysis data for c.440G>A variant in exon 3 of *STK11*: A − Melting peaks; B – Normalized and shifted melting curves; C – Sequencing profile.

DISCUSSION

During the last years it became apparent that breast cancer displays substantial heterogeneity in terms of phenotypic, genetic and clinical characteristics. The disease development and progression correlates with accumulation of genetic and epigenetic alterations, affecting a number of key tumor suppressor genes and proto-oncogenes: *BRCA1*/2, *TP53*, *ATM, CHEK2, STK11, PIK3CA, HER2, PTEN,* etc.

According to Sanger Institute Catalogue of Somatic mutations in Cancer (http://cancer.sanger.ac.uk/cosmic), more than 350 unique mutations in *STK11* have been described (such as nonsense, missense, frameshift, large deletions), established not only in patients with PJS, but also in patients with cancer diseases. Most of these mutations dramatically alter STK11 protein structure leading to impairment of its catalytic functionality and are likely to be of inactivating nature.

In regard to BC, only germline mutations of *STK11* related to hereditary predisposition in PJS were investigated. A relatively small number of investigations has been focused on the

involvement of *STK11* in the sporadic form of BC and according to our knowledge no somatic mutations have been so far acknowledged (BIGNELL *et al*., 1998; FORSTER *et al*., 2000). Our work represents the first HRM-sequencing analysis, concerning *STK11* gene status in Bulgarian patients diagnosed with sporadic breast cancer. Although four *STK11* variants were detected, only two were exonic, of which - one deleterious.

The here observed nonsense variant c.322A>T has not been previously published in the literature but is present in databases and described as a variant of somatic origin detected in lung adenocarcinoma. The mutation found in our study is likely of somatic type of origin as it was not established in the DNA sample from peripheral blood of the same patient and causes premature protein truncation. As predicted by the web-based software the mutation severely damages the normal protein structure and function. However, no statements on its significance for disease pathology could thus far be given.

The missense mutation c.440G>A (rs587780717) was previously described as germline mutation with uncertain clinical significance in Ensembl database and was proposed to be tolerant in INVITAE database with allele frequencies of 0.00006007 according to the “Exome Aggregation Consortium” (ExAC). However, to our knowledge, the variant c.440G>A has never been so far published in the literature. No data concerning the origin of the here established missense variant can be given since the corresponding blood sample of the patient was not available. The site p.147 is not conservative, the alteration does not affect the NLS function and is positioned in the kinase domain of the protein (yet not affecting any of the active sites), leading to substitution of a similar basic, polar and hydrophilic amino acids. The web based prediction programs proposed controversial impact on STK11 protein function. The results obtained through PolyPhen-2 and Swiss Model suggested that the alteration is unlikely to impair STK11 tumor suppressor capability, classifying it as benign. However, SIFT, PROVEAN and LoFtool predicted deleterious effect and defined the alteration as probably damaging. The functional impact and pathological correlation can only be elucidated by means of further detailed functional and clinical assessment.

The intronic variant c.290+36G>T [IVS1+36G>T; intron 1 (+36G→T); rs3764640] is already announced and specified as SNP with unknown clinical significance (dbSNP database) with allele frequencies between 0.32 − 0.35 (1000 Genomes and HapMap Projects) depending on the studied population. The presence of the c.290+36G>T variant is published in a couple of articles, one of which dealing with screening for somatic changes in *STK11* gene in sporadic carcinomas of the colon and testis (AVIZIENYTE *et al*., 1998). The authors identified the particular intronic polymorphism in a colorectal sample; however no potential influence on splicing was discussed. The c.290+36G>T mutation was also observed in investigations on xenografts from sporadic pancreatic cancer (SU *et al*., 1999), in patients with PJS (WESTERMAN *et al*., 1999) as well in head and neck squamous cell carcinomas (QIU *et al*., 2006). The here observed variant was probably of germline type of origin. Although the performed web-based prediction assessment determined that the intronic variant c.290+36G>T has probably damaging effect altering splicing and expression of the gene target, we still cannot conclude that the alteration has pathological consequences. Until detailed functional analysis of the protein is performed and its correlation with disease pathology is studied, the variant remains with uncertain clinical significance.

The observed single base substitution C>A at the 5′ end of intron 9 (c.*16+18C>A) was novel and detected with a relatively high frequency in the studied group of patients. The fact that it was found also in clinically healthy persons suggests that the variant is probably polymorphic. Due to its location, the alteration does not seem to affect the STK11 protein function in general.

However, it is interesting to note that according to the performed web-based prediction assessment, the alteration occurs in a putative intronic splicing silencer (ISS), possibly affecting the regular splicing. Further investigations are required in order to clarify this option.

Our previous genetic studies performed on the same group of patients revealed that *STK11* mutations were found along with genetic mutations in other tumor suppressor genes and proto-oncogenes (BOZHANOV *et al*., 2010; ANGELOVA *et al*., 2012; KRASTEVA *et al*., 2012; KRASTEVA *et al*., 2014). The nonsense *STK11* mutation c.322A>T; p.K108X was coupled with a previously established oncogenic missense change in *PIK3CA* (c.1624G>A; p.Glu542Lys) and with a benign missense mutation in *ATM* (c.2119T>C; p.Ser707Pro). The missense *STK11* mutation c.440G>A; p.Arg147His was combined with an oncogenic missense variant in *PIK3CA* (c.3140A>G; p.His1047Arg). The coexistence of deleterious mutation in *STK11* and *PIK3CA* activating mutation can be explained in view of their involvement in interconnected cellular signaling pathways. As a negative regulator of mTOR kinase activity, inactivation of STK11 results in mTOR overactivity, which in turn leads to stimulation of cell growth and inhibition of apoptosis and autophagy. On the other hand, mTOR can be activated downstream by the positive regulatory role of the PI3K/AKT signaling pathway (KIM and HE, 2013). Therefore, the inactivation of *STK11* and the activation of *PIK3CA* seem to act in the same direction, contributing to cancer development.

In conclusion, to the best of our knowledge this study provides novel data indicating presence of genetic alterations in *STK11* in patients with sporadic breast cancer and is the first screening of the *STK11* genetic status in Bulgarian patients. Irrespective of the observed mutational heterogeneity, a limited number of deleterious alterations were identified. No correlations between the mutational status of *STK11* and clinico-pathological characteristics of the patients were established. In this context, the results suggest that the inactivation of *STK11* through gene mutations is probably a rare event and appears to play a relatively minor role in sporadic breast cancer. However, *STK11* involvement in sporadic breast carcinogenesis could not be excluded, as other (probably epigenetic) mechanisms might be involved in inactivation of the normal STK11 tumor suppressor activity during tumorigenesis.

ACKNOWLEDGMENTS:

Authors express their gratitude to Prof. Dr. Ivan G. Gavrilov who provided tumor and blood samples of the patients under study and to Dr. Maria B. Nacheva who provided clinical data and patients' informed consent. The studied group is a part of a larger group of Bulgarian patients with breast cancer, who were under research within the projects G-1-04/04 and DO-02-310/08. The present work was supported by the grant №BG051PO001-3.3.06-0025, financed by the European Social Fund and Operational Programme Human Resources Development (2007–2013) and co-financed by Bulgarian Ministry of Education and Science. Special gratitude to Petr Pohlreich, M.D., Ph.D., Petra Kleiblova, M.D., Ph.D. and Zdeněk Kleibl, M.D., Ph.D., Laboratory of Oncogenetics, Institute of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague, for their technical support.

Received June 13th, 2016
Accepted January 22th, 2017

REFERENCES

AHMAD, N.N., A.B. CU−UNJIENG, L.A. DONOSO (1995): Modification of standard proteinase K/phenol method for DNA isolation to improve yield and purity from frozen blood. J Med Genet., *32*(2): 129−130.

ANGELOVA, S.G., M.E. KRASTEVA, Z.I. GOSPODINOVA, E.I. GEORGIEVA (2012): *Chek2* gene alterations independently increase the risk of death from breast cancer in Bulgarian patients. Neoplasma, *59*(6): 622−630.

AVIZIENYTE, E., S. ROTH, A. LOUKOLA, A. HEMMINKI, R.A. LOTHE, A.E. STENWIG, S.D. FOSSÅ, R. SALOVAARA, L.A. AALTONEN (1998): Somatic mutations in LKB1 are rare in sporadic colorectal and testicular tumors. Cancer Res., *58*(10): 2087−2090.

BIGNELL, G.R., R. BARFOOT, S. SEAL, N. COLLINS, W. WARREN, M.R. STRATTON (1998): Low frequency of somatic mutations in the *LKB1*/Peutz-Jeghers syndrome gene in sporadic breast cancer. Cancer Res., *58*(7): 1384–1386.

BOZHANOV, S.S., S.G. ANGELOVA, M.E. KRASTEVA, T.L. MARKOV, S.L. CHRISTOVA, I.G. GAVRILOV, E.I. GEORGIEVA (2010): Alterations in *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* genes and their effect in modifying clinicopathological characteristics and overall survival of Bulgarian patients with breast cancer. J. Cancer Res. Clin. Oncol., *136*(11): 1657–1669.

CONNOLLY, D.C., H. KATABUCHI, W.A. CLIBY, K.R. CHO (2000): Somatic Mutations in the *STK11*/*LKB1* Gene Are Uncommon in Rare Gynecological Tumor Types Associated with Peutz-Jegher's Syndrome. Am. J. Pathol., *156*(1): 339–345.

CORRADETTI, M.N., K. INOKI, N. BARDEESY, R.A. DEPINHO, K.L. GUAN (2004): Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev., *18*(13): 1533–1538.

DESMET, F.O., D. HAMROUN, M. LALANDE, G. COLLOD-BÉROUD, M. CLAUSTRES, C. BÉROUD (2009): Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res., *37*(9): e67.

DONG, S.M., K.M. KIM, S.Y. KIM, M.S. SHIN, E.Y. NA, S.H. LEE, W.S. PARK, N.J. YOO, J.J. JANG, C.Y. YOON, J.W. KIM, S.Y. KIM, Y.M. YANG, S.H. KIM, C.S. KIM, J.Y. LEE (1998): Frequent somatic mutations in serine/threonine kinase 11/Peutz-Jeghers syndrome gene in left-sided colon cancer. Cancer Res., *58*(17): 3787−3790.

FENTON, H., B. CARLILE, E.A. MONTGOMERY, H. CARRAWAY, J. HERMAN, F. SAHIN, G.H. SU, P. ARGANI (2006): LKB1 protein expression in human breast cancer. Appl. Immunohistochem Mol. Morphol., *14*(2): 146−153.

FERLAY, J., I. SOERJOMATARAM, M. ERVIK, R. DIKSHIT, S. ESER, C. MATHERS, M. REBELO, D.M. PARKIN, D. FORMAN, F. BRAY (2012): GLOBOCAN v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase, *No. 11*.

FORSTER, L.F., S. DEFRES, D.R. GOUDIE, D.U. BATY, F.A. CAREY (2000): An investigation of the Peutz-Jeghers gene (*LKB1*) in sporadic breast and colon cancers. J. Clin. Pathol., *53*(10): 791−793.

GIARDIELLO, F.M., J.D. BRENSINGER, A.C. TERSMETTE, S.N. GOODMAN, G.M. PETERSEN, S.V. BOOKER, M. CRUZ-CORREA, J.A. OFFERHAUS (2000): Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology, *119*(6): 1447−1453.

GIARDIELLO, F.M., S.B. WELSH, S.R. HAMILTON, G.J. OFFERHAUS, A.M. GITTELSOHN, S.V. BOOKER, A.J. KRUSH, J.H. YARDLEY, G.D. LUK (1987): Increased risk of cancer in the Peutz-Jeghers syndrome. N. Engl. J. Med., *316*(24): 1511−1514.

HEARLE, N., V. SCHUMACHER, F.H. MENKO, S. OLSCHWANG, L.A. BOARDMAN, J.J. GILLE, J.J. KELLER, A.M. WESTERMAN, R.J. SCOTT, W. LIM, J.D. TRIMBATH, F.M. GIARDIELLO, S.B. GRUBER, G.J. OFFERHAUS, F.W. DE ROOIJ, J.H. WILSON, A. HANSMANN, G. MÖSLEIN, B. ROYER-POKORA, T. VOGEL, R.K. PHILLIPS, A.D. SPIGELMAN, R.S. HOULSTON (2006): Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. Clin. Cancer Res., *12*(10): 3209−3215.

HEMMINKI, A., D. MARKIE, I. TOMLINSON, E. AVIZIENYTE, S. ROTH, A. LOUKOLA, G. BIGNELL, W. WARREN, M. AMINOFF, P. HÖGLUND, H. JÄRVINEN, P. KRISTO, K. PELIN, M. RIDANPÄÄ, R. SALOVAARA, T. TORO, W. BODMER, S. OLSCHWANG, A.S. OLSEN, M.R. STRATTON, A. DE LA CHAPELLE, L.A. AALTONEN (1998): A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature, *391*(6663): 184−187.

HEMMINKI, A., I. TOMLINSON, D. MARKIE, H. JÄRVINEN, P. SISTONEN, A.M. BJÖRKQVIST, S. KNUUTILA, R. SALOVAARA, W. BODMER, D. SHIBATA, A. DE LA CHAPELLE, L.A. AALTONEN (1997): Localization of a susceptibility locus for Peutz-Jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis. Nat. Genet., *15*(1): 87-90.

JEGHERS, H., V.A. MCKUSICK, K.H. KATZ (1949): Generalized intestinal polyposis and melanin spots of the oral mucosa, lips and digits; a syndrome of diagnostic significance. N. Engl. J. Med., *241*(25): 993−1005.

JENNE, D.E., H. REIMANN, J. NEZU, W. FRIEDEL, S. LOFF, R. JESCHKE, O. MÜLLER, W. BACK, M. ZIMMER (1998): Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase. Nat. Genet., *18*(1): 38−43.

KARUMAN, P., O. GOZANI, R.D. ODZE, X.C. ZHOU, H. ZHU, R. SHAW, T.P. BRIEN, C.D. BOZZUTO, D. OOI, L.C. CANTLEY, J. YUAN (2001): The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death. Mol. Cell, *7*(6): 1307−1319.

KIM, I., Y.Y. HE (2013): Targeting the AMP-Activated Protein Kinase for Cancer Prevention and Therapy. Front. Oncol., *3*: 175.

KRASTEVA, M.E., G.G. ANTOV, Z.I. GOSPODINOVA, S.G. ANGELOVA, M.B. NACHEVA, E.I. GEORGIEVA, I.G. GAVRILOV (2014): Aberrant promoter methylation in *p53* and *ATM* genes was not associated with sporadic breast carcinogenesis in Bulgarian patients. J. BioSci Biotech., *3*(2): 105−109.

KRASTEVA, M.E., S.S. BOZHANOV, G.G. ANTOV, Z.I. GOSPODINOVA, S.G. ANGELOVA (2012): Breast cancer patients with hypermethylation in the promoter of *BRCA1* gene exhibit favorable clinical status. Neoplasma, *59*(1): 85−91.

PERTEA, M., X. LIN, S.L. SALZBERG (2001): GeneSplicer: a new computational method for splice site prediction. Nucleic Acids Res., *29*(5): 1185−1190.

PEUTZ, J.L.A. (1921): Over een zeer merkwaardige, gecombineerde familiaire pollyposis van de sligmliezen van den tractus intestinalis met die van de neuskeelholte en gepaard met eigenaardige pigmentaties van huid-en slijmvliezen (Very remarkable case of familial polyposis of mucous membrane of intestinal tract and nasopharynx accompanied by peculiar pigmentations of skin and mucous membrane; in Dutch) Nederlandsch Maandschrift voor Geneeskunde, *10*: 134–146.

QIU, W., F. SCHÖNLEBEN, H.M. THAKER, M. GOGGINS, G.H. SU (2006): A novel mutation of *STK11*/*LKB1* gene leads to the loss of cell growth inhibition in head and neck squamous cell carcinoma. Oncogene, *25*(20): 2937−2942.

ROWAN, A., V. BATAILLE, R. MACKIE, E. HEALY, D. BICKNELL, W. BODMER, I. TOMLINSON (1999): Somatic mutations in the Peutz-Jeghers (LKB1/STKII) gene in sporadic malignant melanomas. J. Invest. Dermatol., *112*(4): 509−511.

SANCHEZ-CESPEDES, M., P. PARRELLA, M. ESTELLER, S. NOMOTO, B. TRINK, J.M. ENGLES, W.H. WESTRA, J.G. HERMAN, D. SIDRANSKY (2002): Inactivation of *LKB1*/*STK11* is a common event in adenocarcinomas of the lung. Cancer Res., *62*(13): 3659−3662.

SAPKOTA, G.P., M. DEAK, A. KIELOCH, N. MORRICE, A.A. GOODARZI, C. SMYTHE, Y. SHILOH, S.P. LEES-MILLER, D.R. ALESSI (2002): Ionizing radiation induces ataxia telangiectasia mutated kinase (ATM)-mediated phosphorylation of LKB1/STK11 at Thr-366. Biochem. J., *368*(Pt 2): 507−516.

SHAW, R.J., M. KOSMATKA, N. BARDEESY, R.L. HURLEY, L.A. WITTERS, R.A. DEPINHO, L.C. CANTLEY (2004): The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc. Natl. Acad. Sci. USA, *101*(10): 3329–3335.

SHEN, Z., X.F. WEN, F. LAN, Z.Z. SHEN, Z.M. SHAO (2002): The tumor suppressor gene *LKB1* is associated with prognosis in human breast carcinoma. Clin. Cancer Res., *8*(7): 2085−2090.

SPIGELMAN, A.D., V. MURDAY, R.K. PHILLIPS (1989): Cancer and the Peutz-Jeghers syndrome. Gut., *30*(11): 1588−1590.

SU, G.H., R.H. HRUBAN, R.K. BANSAL, G.S. BOVA, D.J. TANG, M.C. SHEKHER, A.M. WESTERMAN, M.M. ENTIUS, M. GOGGINS, C.J. YEO, S.E. KERN (1999): Germline and somatic mutations of the STK11/LKB1 Peutz-Jeghers gene in pancreatic and biliary cancers. Am. J. Pathol., *154*(6): 1835–1840.

VALERIANOVA, Z., N. DIMITROVA, Y. PETKOVA, L. UZUNOVA, M. YORDANOVA, T. GROZEVA, T. KUNEVA, T. ATANASOV (2015): Cancer Incidence in Bulgaria 2013. Bulgarian National Cancer Registry, Sofia.

WESTERMAN, A.M., M.M. ENTIUS, P.P. BOOR, R. KOOLE, E. DE BAAR, G.J. OFFERHAUS, J. LUBINSKI, D. LINDHOUT, D.J. HALLEY, F.W. DE ROOIJ, J.H. WILSON (1999): Novel mutations in the *LKB1*/*STK11* gene in Dutch Peutz-Jeghers families. Hum. Mutat., *13*(6): 476−481.

WOODS, A., S.R. JOHNSTONE, K. DICKERSON, F.C. LEIPER, L.G. FRYER, D. NEUMANN, U. SCHLATTNER, T. WALLIMANN, M. CARLSON, D. CARLING (2003): LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr. Biol., *13*(22): 2004−2008.

YEO, G., C.B. BURGE (2004): Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. J. Comput. Biol., *11*(2-3): 377−394.

ZHUANG, Z.G., G.H. DI, Z.Z. SHEN, J. DING, Z.M. SHAO (2006): Enhanced Expression of LKB1 in Breast Cancer Cells Attenuates Angiogenesis, Invasion, and Metastatic Potential. Mol. Cancer Res., *4*(11): 843−849.

STK11 GENETSKE MUTACIJE KOD PACIJENATA SA SPORADIČNIM RAKOM DOJKE

Georgi ANTOV[1], Maria KRASTEVA[1], Silvia ANDONOVA[2], Alexey SAVOV[2], Svetla ANGELOVA[1], Lubomir STOILOV[1], Draga TONCHEVA[3]

[1]Laboratorija za dinamiku i stabilnost genoma, Institut za biljnu fiziologiju i genetiku, Bugarska Akademija Nauka, Sofija, Bugarska,
[2] Nacionalna laboratorija za genetiku, UHOG "Maichin dom", Medicinski Univerzitet - Sofija, Sofija, Bugarska,
[3] Departman za medicinsku genetiku, Medicinski fakultet, Medicinski Univerzitet - Sofija, Sofija, Bugarska

Izvod

Germ-lajn mutacije koje utiču na *STK11* (LRG_319) su detaljno proučavane u vezi sa Peutz-Jeghers sindromom, kao predispozicija razvoju više tipova kancera na više lokacija. Iako su somatske mutacije u *STK11* prisutne u više tipova kancera, malo podataka govori o njihovom učešću u razvoju sporadičnog raka dojke. Ovaj rad je usmeren na evaluaciju frekvencije i spektar genetskih promena u *STK11* u grupi Bugarskih pacijenata sa sporadičnim rakom dojke. Ukupno 73 tumora i 22 korespondentna uzorka krvi od pacijenata i 10 uzoraka krvi klinički zdravih kontrola su analizirani. Korišćene su High Resolution Melting analize praćene Sanger sekvenciranjem i bioinformatički alati predviđanja. Sedam pacijenata (9.58%) je imalo *STK11* promene, a samo u dva slučaja (2.74%) su bile eksonske: jedna nonsens c.322A>T; p.K108X (štetna) i jedna missens c.440G>A; p.Arg147His (nepoznatog značaja). Takođe, dve intronske varijante su primećene: c.290+36G>T i c.*16+18C>A (nova). Koliko je nama poznato, ovi rezultati predstavljaju prve podatke koji ukazuju na prisustvo *STK11* promena u pacijentima sa sporadičnim rakom dojke. Ograničen broj otkrivenih štetnih mutacija ukazuje da je mutaciona inaktivacija gena redak slučaj i da verovatno ima manju ulogu u sporadičnoj karcinogenezi dojke.

Primljeno 13. VI. 2016.
Odobreno 22. I.2017.