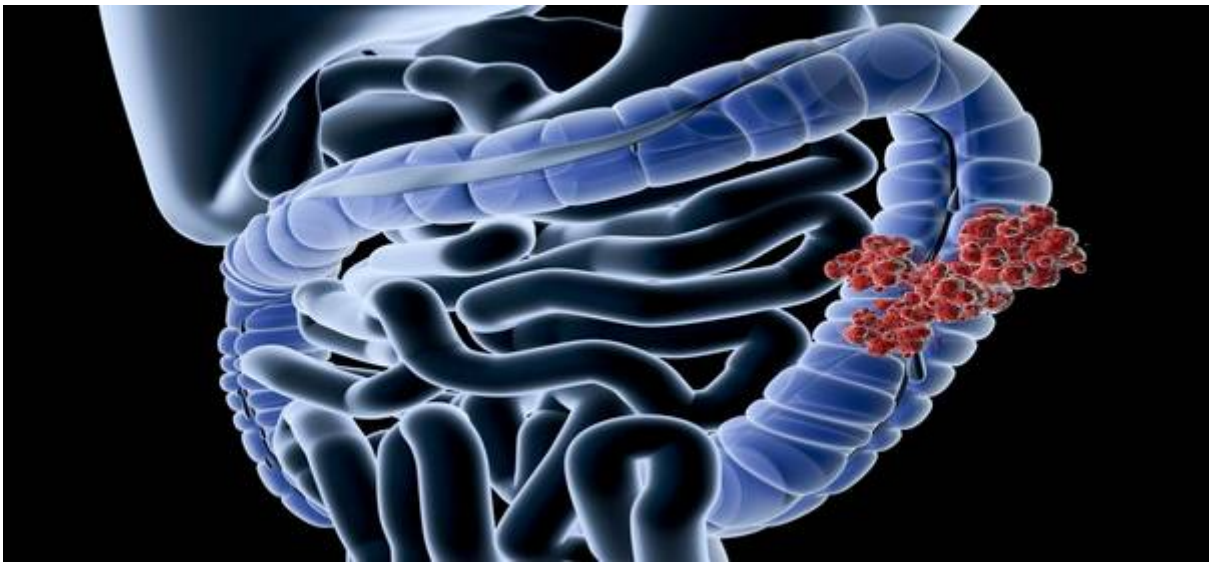
University of Crete, School of Medicine, Graduate program Molecular Basis of Human Disease

Master thesis

LKB1 loss in colorectal cancer patients' samples



SARDAR ALAM

Supervisor: Prof. Ioannis Sougklakos, M.D., Ph.D. Associate Professor of Medical Oncology, School of Medicine, University of Crete, Greece.

INDEX

Abstract:

Lkb1 is considered as a tumor suppressor gene in Puetz-Jegeher syndrome (PJS) as well as in sporadic cancer. Its inactivation due to loss of heterozygosity (LOH) has been reported in some PJS patients, ovarian as well as in lung cancer. We aimed to identify loss of LOH in a relatively large number of colon carcinomas (n=207). We used two different techniques to identify LOH in Lkb1. We identified LOH in 14% colon cancer cases by IHC only. Lkb1 simultaneous nuclear and cytoplasmic (catalytic mutants Lkb1) expression was more common in the metastatic group than the non-metastatic group. We observed two cases having exclusively nuclear staining, and both were from metastatic patients. Our results suggest that LKb1 has a pro-oncogenic role in colon cancer as opposed to its tumor suppressor role because in the metastatic group we observed a higher number of patients that showed positive LKB1 expression than the non-metastatic group. Also, there is a weak positive correlation between normal colon and colon carcinomas on the basis of their Lkb1 expression. We did not detect LOH in 120 samples using microsatellite assay by utilizing 4 different microsatellite markers for the LKB1 locus. We used gene mapping for 120 samples only, in the remaining cases (n=87), we don't have good quality samples.

List of Abbreviations:

PJS	Puetz Jeghers Syndrome
IHC	Immunohsitochemistry
CRC	Colorectal cancer
TGF-β	Transforming growth factor-beta
LOH	Loss of Heterozygosity
STK11	Serine theoronine Kinase 11 (also called LKB1)
Lkb1	Liver Kinase B1
NLS	Nuclear localization signal
EMT	Epithelial-mesenchymal transition
mTOR	Mammalian target of rapamycin
CIN	Chromosomal instability pathway
APC	adenomatous polyposis coli
ATM	Ataxia Telangiectasia mutated kinase
STRAD	STE20-related adaptor (a psuedokinase)
BRG1	Brahma-related gene 1
LM04	LIM domain only 4
PTEN	Phosphate and tension homology deleted on chromosome ten

Identification of loss of heterozygosity of LKB1 in Colorectal cancer patient's samples

1. INTRODUCTION:

1.1 Colorectal Cancer:

According to World Health Organization, colorectal cancer (CRC) is the third most common cancer in the world. It is estimated that more than 1.8 million colorectal cancer new cases and 881,000 deaths will occur in 2018, rendering its rank third in terms of incidence but second in terms of mortality (1). Most of the CRC cases occurred in the developed regions of the world; the highest mortality rates were observed in Central and Eastern Europe. The stage of the disease and the presence of metastasis highly affect the survival rates among the patient group. While the 5-year survival rate is 90% for early-stage patients with localized disease, it is 70% for intermediate (regional invasive tumors) and 10% for advanced-stage patients with distant metastasis. Some factors like age, diet, hereditary polyposis syndrome and inflammatory bowel disease are correlated with the development of CRC (2,3). Metastasis of CRC significantly affects the overall survival among the patients. Distant metastases are present in approximately 25% of patients; diagnosis and resectability of metastasis significantly dictate the outcome (4).

From a biological point of view, CRC represents a heterogeneous disease, associated with clinically meaningful differences in term of disease progression, survival and response to available chemotherapeutic and targeted agents (5). In this regard, three different, but partly overlapping, molecular phenotypes have been recognized reflecting different forms of genomic instability (6). The chromosomal instability pathway (CIN) is the most common phenotype, accounting for up to 85% of all sporadic CRC. Sporadic alterations may be related to different factors such as environmental exposures, diet, hormones, and normal aging (7). Possible mechanisms include alterations in chromosome segregation, telomere dysfunction, and DNA damage response, which affects critical genes such as TP53 and APC (6). CRCs with CIN are characterized by the presence of extensive SCNAs throughout the genome and result in aneuploidic tumors and loss of heterozygosity (7,8). A broadly studied progression model in colorectal cancer correlates the clinicopathological

changes with the genetic alterations in the progression of chromosomally unstable CRC. The initial step in tumorigenesis consists of the adenoma formation associated with the loss of APC (adenomatous polyposis coli). Then adenomas and/or early carcinomas acquire mutations in KRAS, followed by loss of chromosome 18q, SMAD4 and, in the end, TP53 mutations (9).

The microsatellite instability (MSI) phenotype represents 15% of all CRC and is characterized by a deficiency of the DNA mismatch repair system, leading to a large increase in the overall mutation rate. The initial step in tumorigenesis of this phenotype is thought to involve alterations in Wnt signaling, followed by mutations in BRAF gene, that usually occur in place of KRAS mutation in the CIN phenotype. A TP53-independent mechanism of progression to carcinoma seems to be related to the presence of mutations in TGFβ receptor 2 (TGFβR2), insulin-like growth factor 2 receptor (IGF2R) and BAX (9).The MSI phenotype usually exhibits an aberrant DNA methylation profile, leading to the concomitant promoter hypermethylation of multiple genes (9).

Besides the sporadic cases, there are also cases of inherited CRC. The most known hereditary CRC includes the Familial Adenomatous Polyposis (FAP) and the Hereditary Nonpolyposis Colorectal Cancer (HNPCC), MUTYH-associated polyposis (MAP) and hamartomatous polyposis syndromes such as Peutz–Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS), which accounts for approximately 2-4%, 1%, 0.2% and 0.5% of all colorectal cancers (10). Inherited susceptibility should be suspected when CRC is detected in a young patient, in presence of synchronous or metachronous CRC or adenomatous polyps and/or when there is a strong family history of CRC (11).

1.2 Liver Kinase B1 (LKB1):

The LKB1 gene, also known as serine-threonine kinase 11 (STK11), is a tumor suppressor gene which was first identified by Junichi Nezu of Chugai Pharmaceuticals in 1996 in a screen aimed at identifying new kinases (12). LKB1/ STK11 is suggested to act as a tumor suppressor gene in PJS because hamartoma formation in PJS patients with inactivating

LKB1/STK11 germline mutations is associated with somatic loss of the wild-type LKB1/ STK11 allele (13–15). The human LKB1 gene is located on chromosome 19p13.3 and composed of 10 exons, nine of which are coding. LKB1 protein contains 433 amino acids (aa) in human and 436 aa in mouse. LKB1 structure is composed of N- and C-terminal regulatory domains as well as a central catalytic domain. Its catalytic domain spans from aa49 to aa309 with a sequence not closely related to any known protein kinases (16). The structure of LKB1 is shown in figure 1. LKB1 is expressed at varying levels in all fetal and adult tissues examined (12,17). LKB1 encoding an approximately 50 kDa serine/threonine kinase, was first found mutated in Peutz-Jeghers Syndrome (PJS), which is a rare hereditary disease and is characterized by mucocutaneous pigmentation, increased risk of gastrointestinal hamartomatous polyposis as well as benign and malignant tumors (1). The incidence of PJS has been estimated to be between 1/50,000 and 1/200,000 live births (20). Mucocutaneous pigmentations are present in ~95% of patients with PJS, typically arising during the first decade of life, although there have been cases in newborns, while GI polyps arise between the second and third decade of life in 80-100% of patients with PJS (21,22). Patients with PJS with STK11 mutations have a higher risk of developing cancer, with a risk of 81% at the age of 70 years (23).

Germ-line mutations of LKB1 cause the autosomal dominant PJS (12), which significantly increase the risk of developing different cancers esp. colorectal cancer than in the general population (24,25). In addition, germ-line mutations of LKB1 gene are also found in some extraintestinal cancers, such as lung cancer (26,27), breast cancer (28), cervical adenocarcinoma (26), pancreatic and biliary carcinoma (26,29,30), testicular cancer , malignant melanoma (31,32), head and neck squamous cell carcinoma (33) and hepatocellular carcinoma (34). The inactivating nature of PJS mutations together with a frequent loss of the wild-type allele in PJS tumors sustains the notion that *LKB1* is a tumor suppressor.

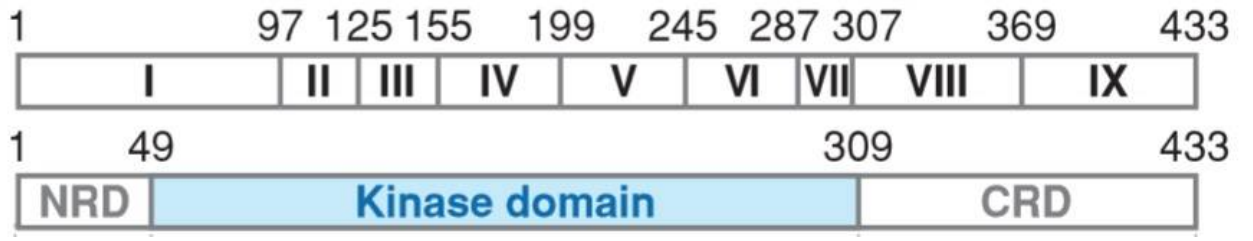


Figure 1: Human LKB1 gene. The genomic organization of the coding sequence of the LKB1 gene is shown on the top, and the functional domains of the protein are shown below (35).

1.3 Expression patterns of LKB1:

In humans there are two LKB1 isoforms, resulting from alternative splicing. The long LKB1 form (50 kDa) is ubiquitously expressed in fetal tissues and, adult tissues with higher expression in epithelia (36). Both isoforms are expressed in humans, but the shorter form (48 kDa) is especially expressed in testis where it has been considered for it's in the role in spermiogenesis. The C-terminus has a unique and shorter amino acid sequence replaced by a shorter and unique amino acid sequence (37). LKB1 is highly conserved, identified to be the strong homolog of mouse LKB1 gene, locating on mouse chromosome 10 and consisting of 10 exons covering about 15 kb (38), Xenopus laevis egg and embryonic kinase 1 (XEEK1), Caenorhabditis elegans (C. elegans) partitioning defective gene 4 (Par-4) and Drosophila LKB1 (14,39,40). Murine Lkb1 shares about 90% identity with human LKB1 and has a conserved prenylation motif) at the carboxyl-terminus directly downstream from a consensus cAMP-dependent protein kinase (PKA) phosphorylation site) (41). During mouse embryonic development, Lkb1 mRNA is predominantly detected in the gastrointestinal tract, lung, and testis (42). However, XEEK1 is reported to be apparently restricted to early embryogenesis (36), and Par-4 protein can be found in the gonads, oocytes and early embryos of C. elegans (39). LKB1 is constitutively active in growing cells (43).In tumors, some malignant tumors express a high level of LKB1, but some cancer cells lost LKB1 expression, and this contradiction may be related to the different process of tumorigenesis.

1.4 Binding Proteins of LKB1:

Endogenous LKB1 can mainly form two complexes which can regulate its stability and kinase activity. Firstly, LKB1 is activated by forming a heterotrimeric complex with STE20-related adaptor (STRAD), an inactive pseudokinase and MO25 (an armadillo repeat scaffolding-like protein) (44,45). STRAD can induce the transportation of LKB1 from the nucleus to the cytoplasm, and activate LKB1 kinase activity through promoting the active conformation of LKB1, while MO25, a 40 kDa scaffolding protein, directly binding to the STRADα carboxyl-terminus, can markedly enhance the binding of STRAD to LKB1 and further stimulate STRAD-induced kinase activity of LKB1 as shown in figure 2 (45–47). At its C terminus, MO25α possesses a deep pocket that binds specifically to Trp-Glu-Phe residues of STRADα, and mutation of residues making up this pocket inhibited the binding of MO25α to STRADα (47).

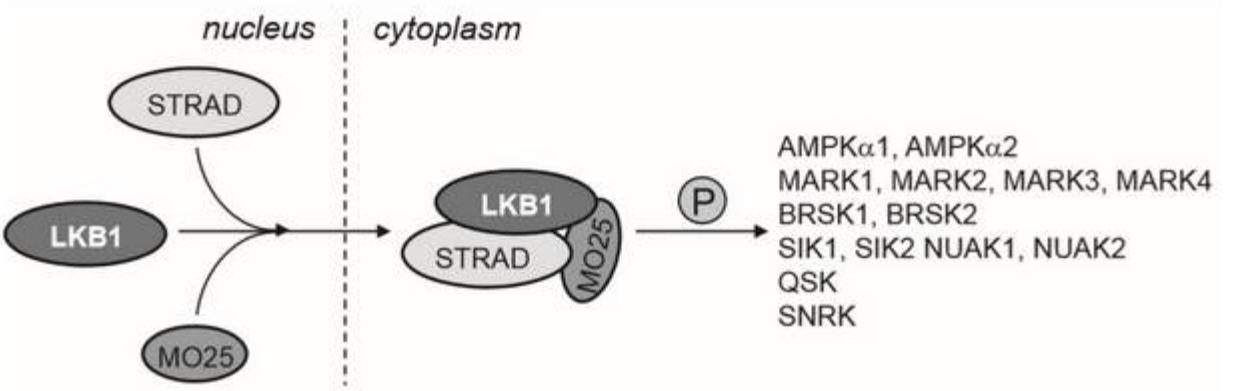


Figure 2: Lacking a nuclear export signal of its own, LKB1 resides in the nucleus. Upon binding to STRADα and MO25, LKB1 is exported to the cytoplasm and becomes catalytically active.

Secondly, LKB1 can also interact with a chaperone complex made up of heat-shock protein 90 (HSP90) and the CDC37, which can stabilize LKB1 in the cytoplasm by protecting it from ubiquitin-dependent degradation (48,49). Furthermore, LKB1–HSP90–CDC37 complex is recently found to function as a repressor of LKB1 kinase activity and disruption of this complex facilitates degradation of LKB1 (50). Thus, LKB1–STRAD–

MO25 and LKB1–HSP90–CDC37 complexes can both stabilize LKB1 with antagonizing effects on LKB1 activity.

Additionally, LKB1 is also reported to interact with other proteins, like p53, AXIN, gene of phosphate and tension homology deleted on chromosome ten (PTEN), LIP1, Brahma-related gene 1 (BRG1), activator of G-protein signaling 3 (AGS3), glycogen synthase kinase 3β (GSK3β), protein kinase ζ (PKCζ), ataxia telangiectasia mutated (ATM), GATA-binding factor 6 (GATA-6)as well as a complex with LIM domain only 4 (LMO4) and LIM domain-binding protein 1 (LDB1) (51–59).

1.5 Subcellular Distribution and Activation of LKB1:

LKB1 has different localizations in mammalian cells. Cytoplasmic localization is known to be significant for the LKB1 growth suppressive function, while it is inactive when its localization restricted to the nucleus (60–62). In samples from PJS patients, wild type LKB1 is distributed in both the nucleus and cytoplasm, while the LKB1 mutant, SL26 (for amino acid deletion), has the normal kinase function, but only accumulates in the nucleus and not detectable in cytoplasm (61,63), further suggesting that cytoplasmic localization of LKB1 is important. Furthermore, the nuclear accumulation of LKB1 may be attributed to its amino-terminal domain (43). Similarly, C. elegans homolog Par4 is reported to be both cytoplasmically and cortically distributed (39). When over-expressed in mammalian cells alone, LKB1 is mostly localized in the nucleus, with a small portion in the cytoplasm (38,61). However, during the apoptosis of cells, LKB1 is found to translocate into mitochondria (51,64). Likewise, Bouchekioua et al, described for the first time a dual localization of LKB1 both in the nucleus and in the cytoplasm in breast tumors and revealed that a nuclear LKB1 expression was inversely correlated with its cytoplasmic expression (65). LKB1 expression is exclusively cytoplasmic in the lung (66) and pancreatic carcinomas (67). However, gastrointestinal hamartomatous polyps from PJS patients (68) and invasive lobular carcinoma and solid papillary ductal carcinoma in situ from patients with breast cancer show both cytoplasmic and nuclear LKB1 expression (69). Similarly, LKB1 was expressed in the nucleus and cytoplasm of tumor cells and HNSCC cell lines. Primary tumors from patients with metastatic carcinoma had decreased nuclear, but not

cytoplasmic, LKB1 compared to patients with nonmetastatic cancer (70). While LKB1 loss has been described in high-grade invasive carcinomas, it is unclear whether this LKB1 loss occurs in the cytoplasm, the nucleus, or both cellular compartments. In addition, LKB1 can be detected on both the plasma membrane and internal membranes in vivo, and this effect is mediated by a functional prenylation motif of LKB1 at the carboxyl-terminus since mutation of Cys433 to an alanine residue of prenylation motif can block membrane localization of LKB1 (41).

The distinct distribution of Lkb1 in the cells may suggest that Lkb1 function is regulated by altering its subcellular localization. Interestingly, it is proposed that Lkb1 may be inactivated by nuclear sequestration and nuclear localization is a common feature of all catalytically inactive Lkb1 alleles, including D303-306 (43,61). In addition to the Lkb1 kinase activity, the nuclear localization requires a nuclear localization signal sequence (NLS) localized on the N-terminus of Lkb1 (38). The N-terminal nuclear localization sequence (NLS) of LKB1 is recognized by Importin-α, which mediates the nuclear translocation (38,71). STRADα translocates LKB1 into the cytoplasm by binding to the export factors CRM1 or Exportin7 and maintains LKB1 in the cytoplasm by outcompeting Importin-α for binding to the NLS of LKB1 (71). Moreover, STRADα and MO25 significantly increase the kinase activity of LKB1 (~9 fold) (44). Activation of the cell polarity program specifically targets LKB1 to the leading edge of the cell where it can interact with downstream binding partners (72). Therefore, activation of specific nuclear and cytoplasmic LKB1 signaling pathways is at least to a certain extent regulated by LKB1 localization.

LKB1 re-localization depends both on its kinase activity and on its ability to interact with STRAD and MO25 (45). Similarly, kinase-deficient PJS mutants, LKB1 point mutations as well as the SL26 PJS mutant, which specifically disrupt the interaction with STRAD and MO25, are retained within the nucleus and are growth arrest defective (63,73). Inconsistent, siRNA-mediated knockdown of endogenous STRADα inhibited the ability of wild-type LKB1 to arrest cell growth(45). In contrast, It is found that the LKB1 C-terminal mutants were translocated to the cytoplasm upon expression of STRAD and exhibited a growth suppressive effect (74). Interestingly, Human and Drosophila LKB1 bind to

phosphatidic acid on cell membrane, which enhances its kinase activity and activation of downstream kinases (at least AMPK, MARKs, and SAD kinases) and activation of AMPK in human cervical carcinoma (HeLa) and melanoma (IGR37) cells depends on membrane-binding of LKB1 (75).

Additional mechanisms may also exist to maintain LKB1 in the cell cytoplasm. For instance, when LKB1 is co-expressed with LKB1-interacting protein 1 (LIP1), a leucine-rich repeat containing cytoplasmic protein, the proportion of cytoplasmic LKB1 dramatically increases in 30% of cells and this indicates LIP1 may regulate LKB1 function by inducing its cytoplasmic localization (52). Recently, Calcium-binding protein 39-like (CAB39L) gene is found to binds and activates STRAD, which further activates LKB1-AMPK-PGC1α axis, which not only inhibits cell cycle progression as well cell invasion and migration as but also promotes cell death (76).

1.6 Regulation of LKB1 Expression:

1.6.1 Epigenetic modification:

In several cancer cell lines, LKB1 gene is found hyper-methylated at the CpG island in its promoter region, and correspondingly, LKB1 transcript cannot be detected, while treatment with the demethylating agent 5-aza-2'-deoxycytidine can restore LKB1 gene expression in these cells (64,77). In addition, one primary colorectal carcinoma and three primary testicular tumors as well as parts of polyps from JPS patients display LKB1 promoter hypermethylation but not in corresponding normal tissues (77). It is consistent that in sporadic colorectal cancer, hypermethylation is found in the LKB1 promoter as well (78). These studies have shown that LKB1 promoter methylation contributed to the silence of the LKB1 gene and LKB1-mediated functions.

1.6.2 Transcriptional Regulation:

LKB1 gene can be regulated by sex hormones, such as androgen and estrogen. Estrogen is reported to regulate LKB1 gene expression through transcriptional regulation,

and ERα binding site is found in the promoter region of LKB1. In MCF-7 breast cancer cells, 17β-estradiol can downregulate both mRNA and protein level of LKB1 through inhibiting LKB1 promoter activity by reducing the binding of ERα to the promoter of LKB1 (79). However, estrogen is also reported to upregulate the expression of LKB1. In mouse and human adipocyte, 17β-estradiol can increase the mRNA level of LKB1 in a dose-dependent manner, and this effect is mediated by ERα (80). Therefore, estrogen-mediated regulation of LKB1 gene expression may be cell-context dependent.

In addition, androgen can also regulate the expression of LKB1 at the transcriptional level. In murine 3T3-L1 and human SGBS adipocytes, testosterone and dihydrotestosterone (DHT) can significantly decrease the mRNA level of LKB1, and this effect is mediated by androgen receptor (80). Interestingly, although androgen can inhibit the mRNA level of LKB1 in adipocytes, there is no androgen receptor element (ARE) found in 2.5 kb promoter region of LKB1 gene (80). This indicates that AR may indirectly regulate the LKB1 gene expression. Besides, fibronectin can also regulate LKB1 expression. In non-small cell lung carcinoma (NSCLC) cells, fibronectin is found to inhibit both the mRNA and protein of LKB1 and LKB1-AMPK signaling (81).

1.6.3 Posttranslational Modification:

Researches find that LKB1 can be phosphorylated, prenylated and ubiquitinated in different conditions. Phosphorylation is the most common posttranslational modification and can either affect the conformation of the phosphorylated protein or create a novel interaction surface for other proteins. At least ten residues of LKB1 have been reported to be phosphorylated, whereof some are autophosphorylation sites (S31, T185, T189, T336, and T402) and others residues are phosphorylated by other kinases (S307, S325, S334, T366, S399 and S428 (82,83). LKB1 can also be phosphorylated at Ser31, Ser325, and Thr366 when expressed in HEK-293 cells, but its phosphorylation affects neither its nuclear localization nor its catalytic activity in vitro (84). In contrast, the phospho-mimetic LKB1 T336E mutant prevented LKB1 from suppressing the growth of a human malignant melanoma cell line, suggesting an autoinhibitory function of this autophosphorylation site (84).

Several kinases, such as protein kinase A (PKA), PKC, AKT, and ATM, have been reported to phosphorylate LKB1 protein in animal models (41,85,86). PKCζ can activate LKB1 through phosphorylation of Ser428 and S307 of LKB1, which enhances the nuclear export of LKB1, resulting in binding of LKB1 with AMPK and subsequent AMPK phosphorylation and activation (83,87). Loss of the phosphorylation sites S307 and S428 attenuates the association of LKB1 with STRADα and the export factor CRM1 and thus decreases the cytoplasmic accumulation of LKB1 in an adenocarcinoma cell line (83). Consequentially, the phosphorylation-deficient LKB1 S307A mutant has a low capability to suppress angiogenesis and cannot efficiently protect cells from apoptosis (83). Phosphorylation of S399 in LKB1S promotes its nuclear export followed by the activation of AMPK and is therefore thought to be the functional equivalent to S428 in LKB1 (88). Other studies find that Thr366 of LKB1 can be phosphorylated by ATM 73, and activators of extracellular signal-regulated kinase 1/2 (ERK1/2) can induce the phosphorylation of endogenously expressed LKB1 at Ser431 (89).

The carboxyl-terminus of murine Lkb1 can be prenylated in vivo, and PKA-mediated LKB1 prenylation, but not phosphorylation, leads to membrane distribution of LKB1 (41).

Except for phosphorylation and prenylation, LKB1 can be posttranslationally modified by ubiquitination and deacetylation. SIRT1, a member of the sirtuin family of proteins, functions as a negative regulator for LKB1/AMPK signaling in primary endothelial cells by promoting deacetylation, ubiquitination and proteasome-mediated degradation of LKB1 (90). However, SIRT1 can also activate LKB1 by deacetylating and subsequently increasing the phosphorylation and activity of LKB1. In 293T cells, SIRT1 over-expression abates lysine acetylation of LKB1 and concomitantly enhances its activity and finally activated AMPK downstream signaling (91). These findings suggest that LKB1 is a target of SIRT1-mediated deacetylation, which can further influence its kinase activity and downstream signaling.

1.7 Biological functions of LKB1:

1.7.1 Regulation of AMPK and related kinases

LKB1 has been termed a master kinase based on its ability to control the activity of the 14 members of the AMPK-related family of serine-threonine kinases through direct phosphorylation of a threonine residue in their activation loops (92). In complex with the pseudokinase STRAD and the scaffolding protein MO25, LKB1 phosphorylates and activates AMPK family kinases, which mediate many cellular processes. LKB1 is the major upstream kinase that phosphorylates and activates AMPK (93). Cells lacking LKB1 have decreased the activity of these AMPK family kinases whereas restoration of wild-type LKB1 rescues their catalytic activity. AMPK, the widely explored among these, is central to the maintenance of cellular energy homeostasis, coordinating catabolic and anabolic cellular activities in normal and transformed cells (94–96). Activated AMPK phosphorylates a number of proteins resulting in a decrease in ATP-consuming processes and an increase in ATP production through inhibition of protein synthesis, fatty acid, and glucose metabolism and enhancement of glucose transport (96). AMPK turns off mTOR (mammalian target of rapamycin) by signaling to tuberous sclerosis (TSC2/TSC1) tumor suppressor complex, as well by directly phosphorylating the mTOR-binding partner, raptor (regulatory associated protein of mTOR) (97–99). TSC2/TSC1, in response, inactivates the small GTPase Rheb (Ras homolog enriched in brain), which positively signals to mTOR. In some cell types, PKC ζ contributes LKB1– AMPK signaling, possibly by phosphorylating LKB1 and promoting its cytoplasmic translocation and interaction with AMPK (100,101).

Importantly, this role of LKB1 in energy metabolism has been demonstrated in vivo in the mouse and C. elegans. Somatic deletion of Lkb1 in the mouse skeletal muscle produces defects in glucose uptake and loss of AMPK activation (102,103). Liver-specific Lkb1 deletion also causes metabolic defects and loss of activity of AMPK and increased mTOR signaling (104).

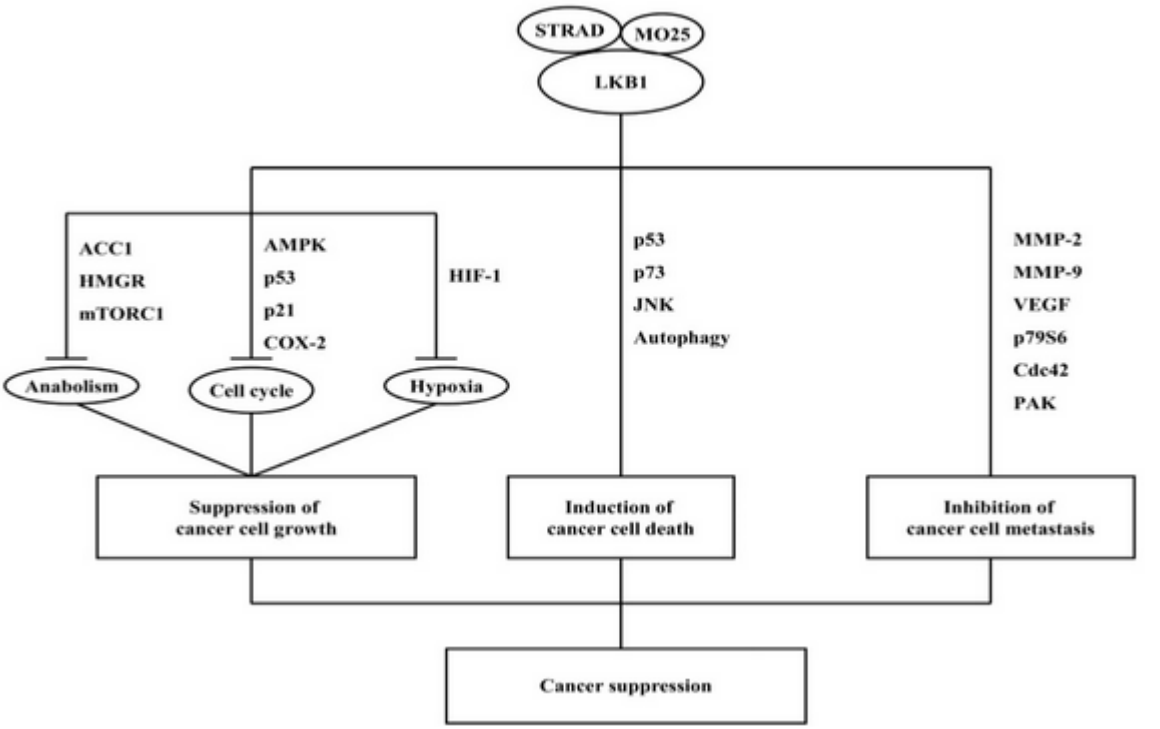


Figure 3: A diagram describing the potential mechanisms of liver kinase B1 (LKB1)-mediated cancer suppression (105)

1.7.2 Apoptosis and Cell Cycle Arrest:

LKB1 has been found to associate with p53 physically as well as regulate specific p53-dependent apoptosis pathways and one study indicated the absence of apoptosis in polyps from patients with PJS (51). Recent reports have shown that the cytoplasmic localization of LKB1 is sufficient to induce arrest in the G1 phase of the cell cycle (44). In addition, LKB1 has been reported to interact with and phosphorylate PTEN, another tumor suppressor that has lipid phosphatase activity and that inhibits cell proliferation and survival (53). LKB1 has also been found to suppress the antiapoptotic factors, such as STAT3, JNK, c-myc, k-ras, MAPK, and cyclooxygenase-2, and to inhibit cell survival (35,106,107). Similarly, LKB1's putative downstream targets, such as Brg1, p21, and p27, have been suggested to mediate LKB1 dependent cell cycle arrest (54,61,108). These observations suggest that the tumor suppressive function of LKB1 may result from the inhibition of cell cycle progression.

1.7.3 Maintenance of Genome Stability:

Analysis of the LKB1 protein sequence and structure has shown that LKB1 Thr 363 (Thr 366 in mouse) lies in an optimal phosphorylation motif for the phosphoinositide 3kinase-like kinases, such as DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated kinase (ATM), and ATM- and rad3-related kinase (ATR). These kinases act as DNA damage sensors, functioning upstream of DNA damage and mediating DNA repair (109,110). Moreover, in a vitro assay, it was demonstrated that wild-type ATM displays a DNA damage-induced association with LKB1, BRCA1, and p53 (57). Additionally, Alessi's group reported that the phosphorylation of LKB1 at Thr 366 was triggered following the exposure of cells to IR and that DNA damage-activated ATM kinase mediated this phosphorylation (89). LKB1 is also involved in DNA damage checkpoint regulation, in which cells lacking LKB1 accumulate more DNA-damage than control cells (111). LKB1 depletion induced the formation of chromosome breaks and radials (112). These results suggest that LKB1-AMPK signaling may contribute to DNA damage repair and play a role in the maintenance of genome stability.

1.7.4 Inhibition of Tumor Progression and Metastasis as well as its emerging pro-oncogenic role:

The importance of LKB1 in tumor suppression was recently further highlighted by its function in the suppression of cancer invasion and metastasis (113–116). In the In vitro studies, investigations demonstrated that LKB1 knockdown increases cell motility and invasiveness, and induces the expression of several mesenchymal marker proteins accompanied by the expression of ZEB1, a transcriptional repressor for E-cadherin and an inducer for epithelial-mesenchymal transition (EMT), which is a critical phenotypic alteration initiating the invasion and metastasis of cancer cells (113–116). Cancer cells may become resistant to anoikis and consequently display anchorage-independent growth. It was found that LKB1 involves in p53-dependent anoikis by regulating salt-inducible kinase (SIK1), an AMPK family member (117). SIK1 was required for LKB1 to promote p53-dependent anoikis and suppress anchorage-independent growth and invasion.

In a range of mouse models and clinical analyses, LKB1 inactivation is consistently found to be associated with aggressive invasive and metastatic growth. In the aforementioned LKB1-SIK1-p53 anoikis model (117), loss of LKB1 promotes metastatic spread and survival of cells as micrometastases in the lungs. In a K-ras driven mouse model of lung cancer, LKB1 inactivation provided the strongest cooperation in terms of tumor latency and frequency of metastasis as compared with classic tumor suppressors such as p53 and p16 (113). Consistently, Castrillon's group showed that LKB1 mutations in primary cervical cancers are linked to accelerated disease progression and death (118), and decreased LKB1 protein expression in endometrial cancers correlates with a higher grade and stage (119). Strikingly, female mice with homozygous endometrial Lkb1 inactivation did not harbor discrete endometrial neoplasms, but instead underwent a diffuse malignant transformation of their entire endometrium with rapid extrauterine death, suggesting that Lkb1 inactivation was sufficient to promote the development of invasive endometrial cancer (120).

In addition, some studies have shown the oncogenic role of Lkb1 against its pre-established notion of tumor suppression. For instance, LKB1 pro-oncogenic activity triggers cell survival in circulating tumor cells (121). Lkb1 is polyubiquitinated by Skp2-SCF ubiquitin ligase to activate Lkb1-AMPK axis, and surprisingly oncogenic H-Ras promotes the polyubiquitination and activation of LKB1 (122). Similarly, recently Guo et al, showed that AMPK promotes the survival of colorectal cancer stem cells (123), and as Lkb1 is its upstream kinase, so AMPK activation probably by Lkb1 enhance the survival of cancer stem cells. Additionally, Ng TL et al demonstrated that the LKB1/AMPK pathway protect epithelial cells from anoikis once they detach from a surface (124) and that LKB1 has a central role in arbitrating anoikis resistance especially in ovarian cancer (125). Some other studies also propose the pro-oncogenic role of Lkb1 in the context of AMPK activation (126,127). Thus, the pro-oncogenic role of Lkb1 has to be considered in addition to its tumor suppressor role.

1.7.5 Regulates cancer cell invasion and migration:

Tumor cell invasion and metastasis is a complex process that results from interaction and crosstalk between multiple pathways. The LKB1/AMPK pathway regulates invasion and metastasis via diversified signaling pathways, such as NF-κB, AKT, forkhead box O3 (Foxo3a), TGF-β and mTOR (128). Emerging evidence indicates that LKB1/AMPK inhibits tumor invasion and migration through downregulation of the downstream factors of TGF-β signalings, such as MMPs and Snail (129,130). Another study revealed that LKB1 limits the migration and the invasive capacity of breast cancer cell lines by decreasing levels of secreted matrix metalloproteinases and downregulating the expression of angiogenesis factors, such as VEGF, both in vitro and in vivo (131). LKB1 is also involved in other signaling pathways as well, for instance, the TGF-β pathway. In this pathway, LKB1 forms a complex with LIP-1 and the transcription factor Smad-4, inhibiting the translocation of Smad-4 to the nucleus and thus regulating the TGF-β pathway (48). Indeed, It was reported that AMPK inhibited TGF-β-induced EMT in HK-2 cells (132). The TGF-β signaling pathway promotes cancer metastasis while the LKB1/AMPK pathway negatively regulates the progression of cancer. hence, the regulation of TGF-β signaling may be a vital mechanism by which LKB1/AMPK inhibits tumor metastasis.

1.8 LKB1 Inactivation causes:

1.8.1 LKB1 Mutations:

It was reported that germline mutations of LKB1 occur in 80% PJS patients (133,134). Gastrointestinal tumors are the most commonly diagnosed tumors in PJS patients, but the risk of developing cancer from other origins is also markedly higher, such as cancers from breast, pancreas, and gonad, etc as shown in figure 3 (135,136). To date, more than 250 different mutations in LKB1 have been identified in PJS patients and sporadic cancers according to the Sanger Institute Catalogue of Somatic mutations in Cancer website (https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=KLKB1#distribution). Almost 80% of them are missense or nonsense mutations, which mostly lead to truncations of the catalytic domain and impair LKB1 catalytic activity. However, there are also a significant number of point mutations, which are located in the kinase domain and in the C-terminal noncatalytic

region which provide evidence that the elimination of the kinase activity of LKB1 is probably responsible for the development of the PJS phenotypes (35,137). Truncating variants in STK11 are thought to predispose to a more severe phenotype. Missense variants are generally considered less severe than truncating variants (138). Mutations in the LKB1 tumor suppressor are frequently detected in tumors from Caucasian but not Asian lung cancer patients (139). It was found that C-terminal mutations neither disrupt LKB1 kinase activity nor interfere with LKB1-induced growth arrest but these naturally occurring mutations lessened LKB1-mediated activation of the AMP-activated protein kinase (AMPK) and impaired downstream signaling (74). Furthermore, C-terminal mutations compromise LKB1 ability to establish and maintain the polarity of both intestinal epithelial cells and migrating astrocytes (74).

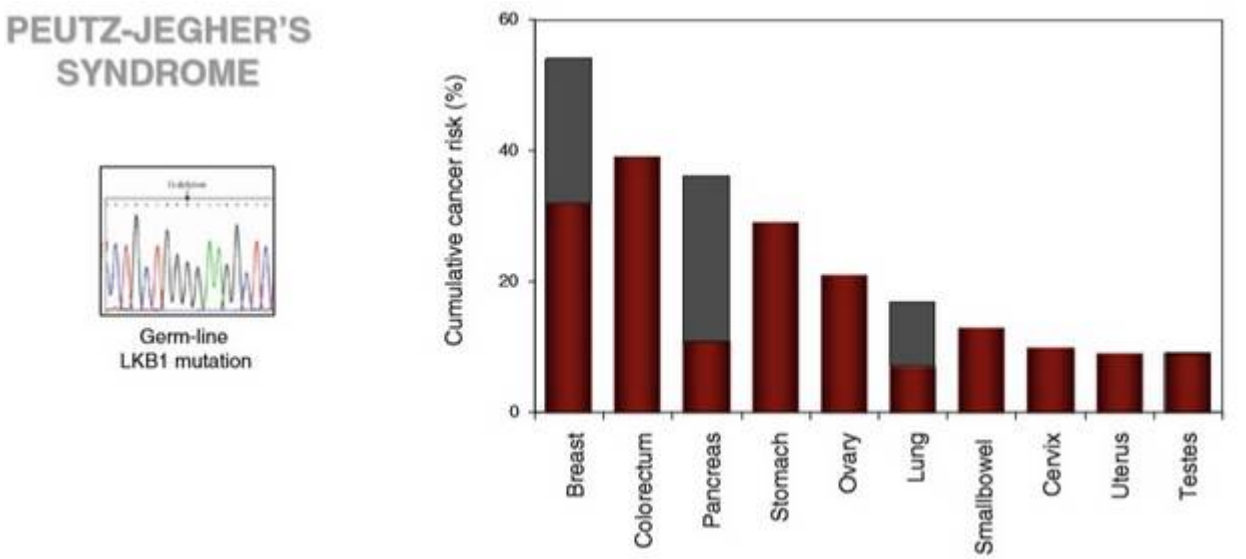


Figure 4:Cumulative cancer risk of patients with the Peutz Jegher's syndrome (25). The bars indicate the percentage for the indicated tumor type. The black bars indicate different percentage ranges in different studies.

Although tumor-specific LKB1 alterations have been identified in many tumor types, their

frequency is relatively low with the exception of nonsmall cell lung cancer (NSCLC), gastrointestinal tract tumors, and cervical cancer. In NSCLC, 30% of the patients are reported to be LKB1 inactivate (27,143). The differences for the cancer distribution patterns between PJS and sporadic cancer in LKB1 mutations remain unclear. As the cancer genome program becomes complete, the accurate LKB1 mutation pattern may be revealed.

1.8.2 Epigenetic inactivation of LKB1:

Since LKB1 somatic mutations in sporadic cancers have been relatively rare, it has been suggested that another mechanism may be important for inactivating LKB1. This has been supported by demonstrating LKB1 promoter methylation and mRNA down-regulation in primary tumors and cell lines (77,78). It was found that promoter hypermethylation and allelic loss of the LKB1/STK11 gene are rare events in sporadic colorectal cancer in Caucasian patients as well as LOH at 19p13.3 is associated with advanced tumor stage and left-sided location (142). Thus, epigenetics play a role in Lkb1 inactivation.

1.8.3 Loss of LKB1 heterozygosity:

It is also one of the mechanisms of LKB1 inactivation. The loss of one allele of a genetic locus can have multiple possible functional effects including haploinsufficiency, loss of gene expression and is the second ‘hit’ that unmasks a recessive tumor suppressor gene. LOH can be caused by mitotic errors, chromothripsis, gene conversion and inappropriate repair of DNA breaks (143). Loss of heterozygosity can be identified by using polymorphic markers, such as microsatellites or single-nucleotide polymorphisms (SNPs). Entius and Miyaki suggested that LOH of LKB1 might be less frequent in PJS polyps (144,145). Similarly, homozygous deletions of LKB1 have been frequently reported in NSCLC (146,147). Interestingly, Rowan studied LOH negative PJS polyp by in situ hybridization and demonstrated LKB1 mRNA expression throughout the polyp, suggesting that some polyps retain LKB1 mRNA expression (36).

When comparing the frequency of LOH in polyps to carcinomas from PJS patients,

LOH was detected in between 25% and 38% of polyps and between 64% and 100% of carcinomas, respectively (144,145,148). This suggests that bi-allelic loss of LKB1 is not necessary for hamartomatous polyp development, but favors progression to carcinoma. Therefore, LKB1 is suggested to act as a haploinsufficient tumor suppressor gene. The haploinsufficiency of Lkb1 has been confirmed in mice, where neither loss of the wild-type Lkb1 allele nor loss of expression of Lkb1 could be detected in most gastrointestinal polyps of heterozygous Lkb1 knockout mice (149–151). These results suggest that partial loss of Lkb1 is sufficient for tumor development. haploinsufficient tumor suppressor genes usually evoke their role in tumorigenesis in the context of additional oncogenic triggers. In the case of Lkb1, it has been shown that additional loss of Pten or p53, or additional oncogenic activation of Kras synergizes with a Lkb1 loss in order to promote tumor formation (152–155). In a subset of gastrointestinal hamartomas of heterozygous Lkb1 knockout mice loss of the wildtype Lkb1 allele or Lkb1 expression could be detected specifically in the epithelial compartment, suggesting that Lkb1 exerts its tumor suppressive functions mainly in the epithelium (149). However, myofibroblast-specific loss of Lkb1 in mice has been shown to be sufficient for gastrointestinal hamartoma development, indicating that Lkb1 suppresses tumor formation through signaling in mesenchymal cells (156). No phenotypical differences were observed between polyps of mice with mono-allelic or bi-allelic Lkb1 deletion, suggesting that also in stromal cells Lkb1 acts as a haploinsufficient tumor suppressor (156). So, in human cancers, the frequency of single-copy allelic loss at chromosome 19p spanning the Lkb1 locus without inactivation of the second copy of Lkb1 may reflect a haploinsufficient tumor suppressor function in carcinomas (113).

LKB1 inactivation occurs in 30% of lung cancer and out of which more than 50% inactivation occurs due to LOH (146). In addition, LOH was found in brain metastasis, breast cancer and lung metastasis (157), although LOH was also noted in colorectal samples in the same research, it has lower significance because LOH was noted in only three samples. So that's why we carried out LOH study in a relatively good amount of colorectal samples (n=207).

2. OBJECTIVE:

To identify loss of heterozygosity (LOH) by using PCR based microsatellite assay and Immunohistochemistry techniques

3. MATERIALS AND METHODS:

3.1 Tumor Samples:

The samples of colorectal carcinomas were collected between 2003 to 2010 from the Medical Oncology Department of the University Hospital of Heraklion, Crete, Greece and were stored as formalin-fixed, paraffin-embedded (FFPE) in Lab of Tumor biology, medical school, University of Crete. Patients consented to an Institutional Review Board (IRB)-approved protocol allowing collection of specimens and clinical data. All the Tumours were microdissected using a fine scalpel blade to enrich for neoplastic tissue, but had, in any event, been selected for a lack of contaminating normal tissue. The tumor content of each of the specimens was confirmed to be sufficient for LOH assay and IHC analyses.

3.2 DNA Extraction:

DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue by MasterPure™ DNA Purification Kit (Epicentre, Illumina Company, Cat. No. MCD85201).

Procedure:

Firstly, remove 15 µl thick paraffin embedded tissue and place 15 mg of it in an appropriate tube. Then, add 2 ml of Xylene to extract the paraffin and incubate at room temp for ten 10 minutes. After that, add 2 ml of 100% ethanol and incubate at room temp until the ethanol aspirate. Add 300 µl of tissue and cell lysis solution containing the Proteinase K (2 µl to 300 µl of tissue and cell lysis solution) to the sample and mix. Incubate at 65 degree Celsius for 30 minutes: followed by a brief (10 seconds) vertex mix and add I µl of RNase A to the sample.

Then, proceed with the DNA precipitation. Add 175 µl of MPC Protein Precipitation Regeant to 300 µl of lysed sample and vortex mix vigorously for 10 seconds. Pellet the debris by centrifugation at 4 degree Celsius for 10 minutes at 10000 rpm in a microcentrifuge. Then transfer the supernatant to a clean microcentrifuge tube and discard the pellet. After that, add 500 µl of isopropanol to the recovered supernatant. Pellet the DNA by centrifugation and carefully pour off the isopropanol. Rinse twice with 70% ethanol. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with

a pipet and resuspend the DNA in 35 µl of TE buffer. DNA was quantified using the NanoDrop ND-1000 v3.3 (ThermoFisher Scientific, Waltham, Massachusetts, USA) equipment and the samples were stored at -20°C until their use.

3.3 PCR based Microsatellite Assay (Gene mapping):

We screened 120 colorectal carcinomas for LOH at the LKB1/STK11 locus, using highly polymorphic fluorescent microsatellite markers DI9S886, D19S883 (located telomeric to the LKB1/STK11 gene), D19S878 and D19S565 (located centromeric to the LKB1/STK11 gene). The markers have been used previously in PJS as well as for other tumors (146,157). PCR primer sequences D19S878 (Forward GCCTGGGCGACAGAGAAT and Reverse GGTTGCCCGCAGAGTG), D19 S883 (Forward TTACGGGCAGGACTTGAGTGG and Reverse GTCACGAGTTCACAAGTTTGG), D19S886 (Forward TGGATCTACACTCCGGC and Reverse ATTTTACTGGCTGGCACTTG) and D19S565 (Forward GTGATTGCACCACGGG and Reverse TCAAGTCATTGGGTTGGC) were used in our experiment. Primer specificity was confirmed by direct sequencing of the microsatellite fragments. Lung cancer cell lines A549, H460, and H1299 were used as a positive control for LOH as they have loss of exons in their chromosomal region of LKB1 (27,158). Similarly, lung cancer cell line HCC827 was used as a negative control as it does not have mutation/allele loss at chromosome 19p region (159,160). At least two microsatellites were analyzed for each tumor sample. For analysis of allelic deletions by LOH, 30ng of DNA was PCR-amplified in a total volume of 25µl containing 0.2µM each primer (D19S886 with Fam and D19S565 with Hex labeled), 2.0mM MgCl2, 200µM dNTPs, 2.5µl of 10×PCR buffer (Perkin Elmer Corp.), and l.5U of Taq polymerase (Perkin Elmer). PCRs were performed in a Gene Amp 9600 thermocycler (Perkin Elmer) with an initial denaturation step (95 °C for 4min) and a total of 27 cycles of denaturation (94°C for 1min), annealing (58 °C for 1min), and extension (72 °C for 1min), followed by 7min at 72 °C (final elongation).

About 0·1–0·2 µl of each PCR product was mixed with 0·5 µl of TAMRA350 size standard (ABI) and 1 µl of formamide loading buffer. This mixture was heated to 96 C for 5 min and electrophoresed on a 4·5 % acrylamide sequencing gel on an ABI Prism 3130

(ThermoFisher Scientific, USA) semiautomated sequencer for 2-4 h. Results were analyzed using Genescan (ABI) software. In informative (heterozygous) cases, allele loss was scored if the area under one allele peak was reduced in a tumor to less than 0·5 of its value in the normal DNA (relative to the other allele), after taking account of baseline fluorescence levels and 'stutter' bands. This threshold for allele loss allows for the possibility of contaminating normal tissue or other tumor clones in some samples.

3.4 Immunohistochemistry:

In our experiment, we used Thermo Scientific™ UltraVision™ Quanto Detection System HRP for IHC of Lkb1. The bold words show the kit components.

Procedure:

First we cut 5-µm sections from the paraffin blocks, then dewax as well as rehydrate it with xylene and graded alcohol, respectively. The slides were treated in sodium citrate buffer and steamed for 20 minutes at 90 C. To reduce nonspecific background staining due to endogenous peroxidase, incubate the slides in **Hydrogen Peroxide Block** for 10 minutes. Apply **Ultra Vision protein Block** and incubate for 5 minutes to block nonspecific background staining. Then apply a primary polyclonal antibody to LKB1 (1:100 dilution, Thermo scientific, USA) and incubated for I hour. Wash the slides with buffer. Apply **Primary Antibody Amplifier Quanto** and incubate for 10 min. After wash buffer step, apply **HRP Polymer Quanto** and incubate for 10 min. Then add 30 µl (1 drop) **DAB Quanto Chromogen** to 1 ml **of DAB Quanto Substrate**, mix by swirling and apply to tissue. Incubate for 5 minutes. After washing, counterstain the slides with methylene blue and coverslip using a permanent mounting media.

Adult seminiferous tubules of the testis were used as a positive control as it shows the highest levels of LKB1 expression (36,161). Negative controls were obtained by performing all of the IHC steps but leaving out the primary antibody. IHC staining intensity was measured using a scaling system of 0 (no expression), 1, 2 and 3 (highest expression) blinded by a pathologist. To control for differences in LKB1 expression levels and subcellular localization, a

weighted index (WI) was determined in both the nucleus and the cytoplasm using the equation WI = % tumor stain X intensity score as used in another study (162).

Moreover, the following criteria were used to guide the measurement of LKB1 staining: (a) The LKB1 staining was considered positive when the intensity in the neoplasms matched or exceeded the staining intensity of the testis slide (b) Conversely, LKB1 staining was classified as negative if the staining intensity in the neoplasms was reduced relative to the testis slides. The staining of LKb1 was interpreted without knowledge of its genetic status.

3.5 Statistical Analysis:

The difference of percentage between LKB1 exclusively cytoplasmic group versus both nuclear and cytoplasmic expression was determined using chi-square test. The difference between mean ages between the two groups was calculated by using independent sample T-test. Similarly, Kaplan Meier survival estimates were generated to test whether the overall survival and Disease-free interval (DFI) were different for those patients who demonstrated cytoplasmic expression from those who had both cytoplasmic as well as a nuclear expression at the same time and intergroup differences were determined using log-rank test. Kaplan Meier curves were also used in case of LKB1 positive versus negative expression in the patients. $P<0.05$ was considered to indicate a statistically significant result. All the statistical analyses were conducted using SPPS version 25 (IBM USA).

4. RESULTS:

4.1 Microsatellite analysis:

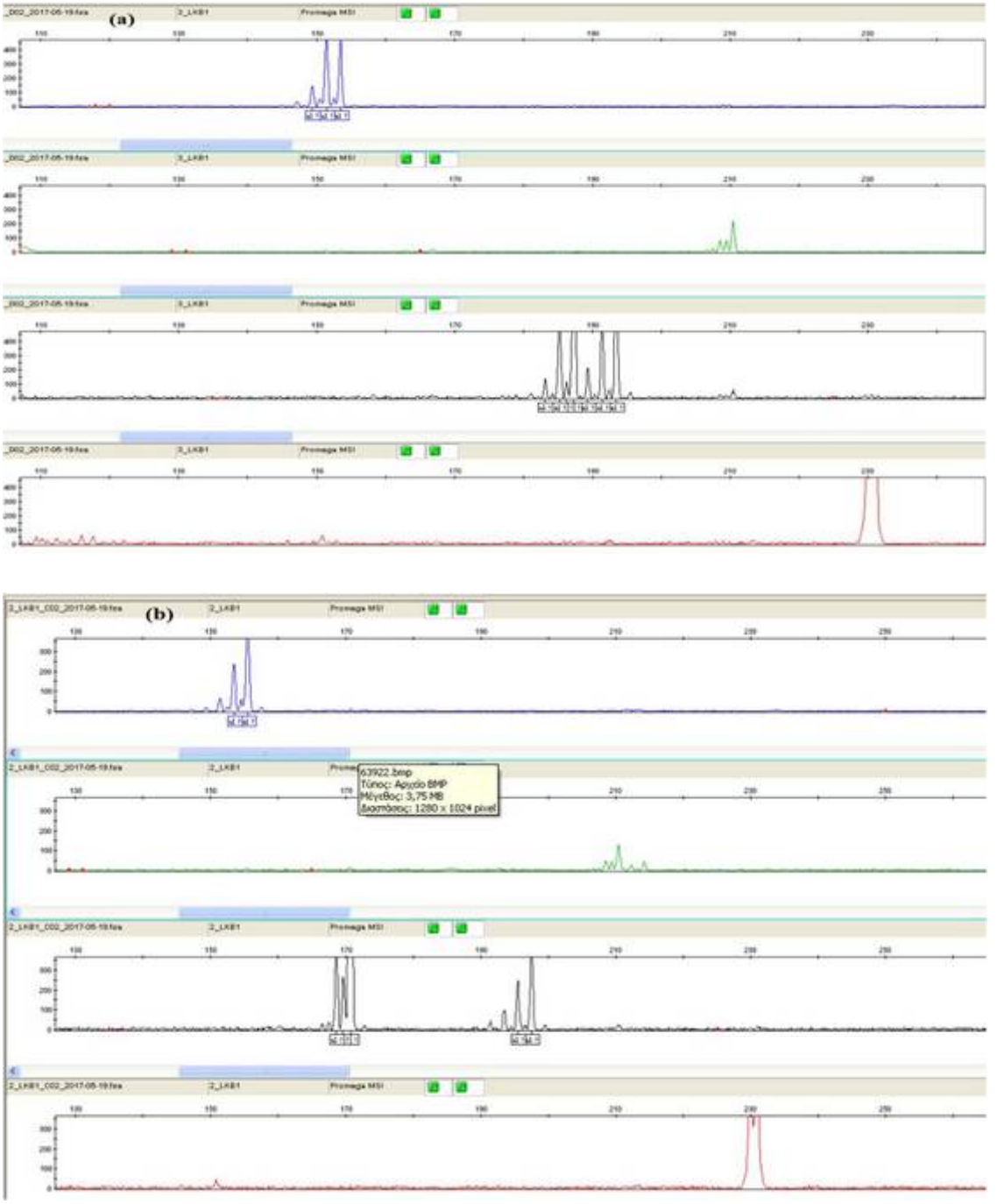
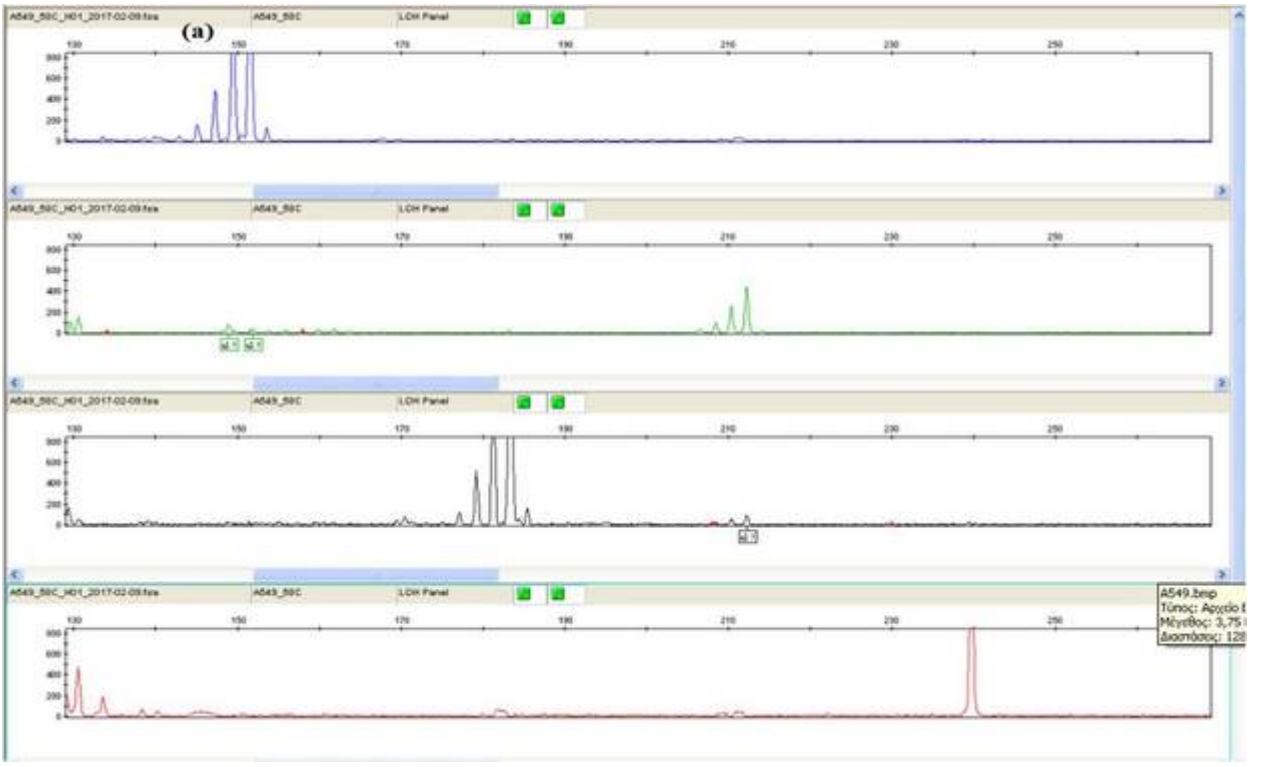
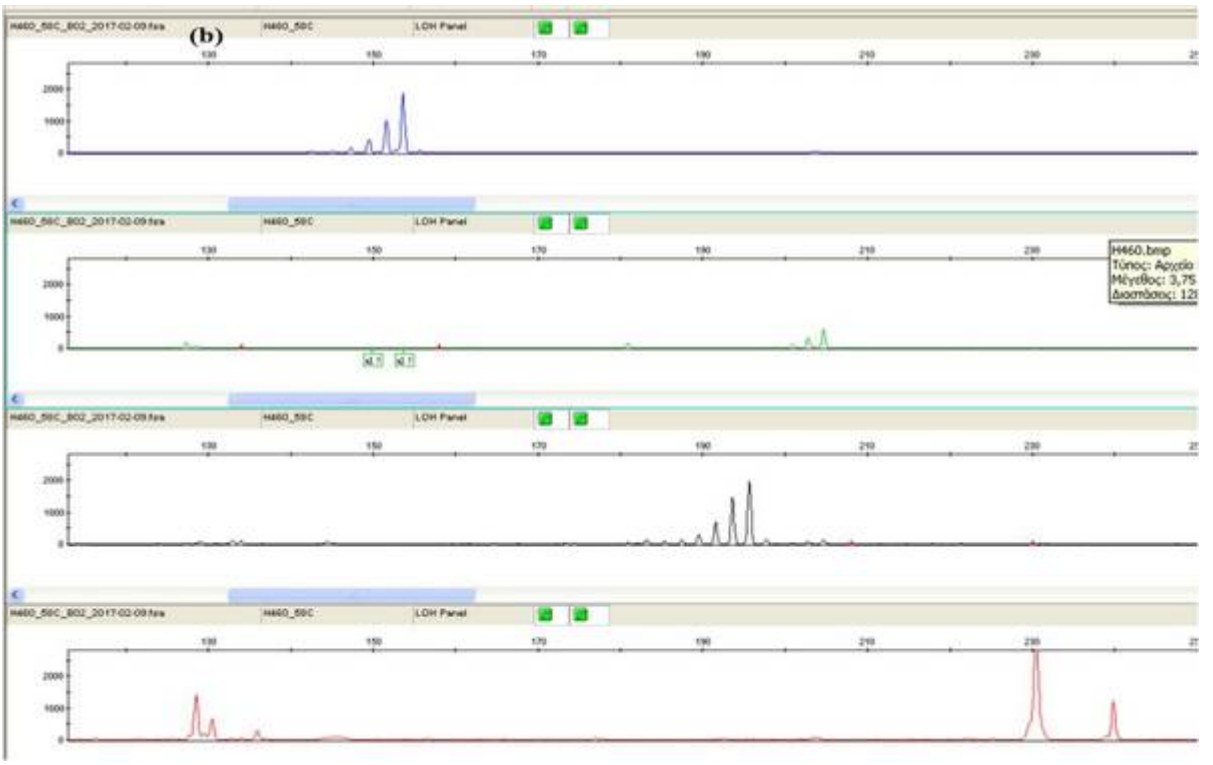


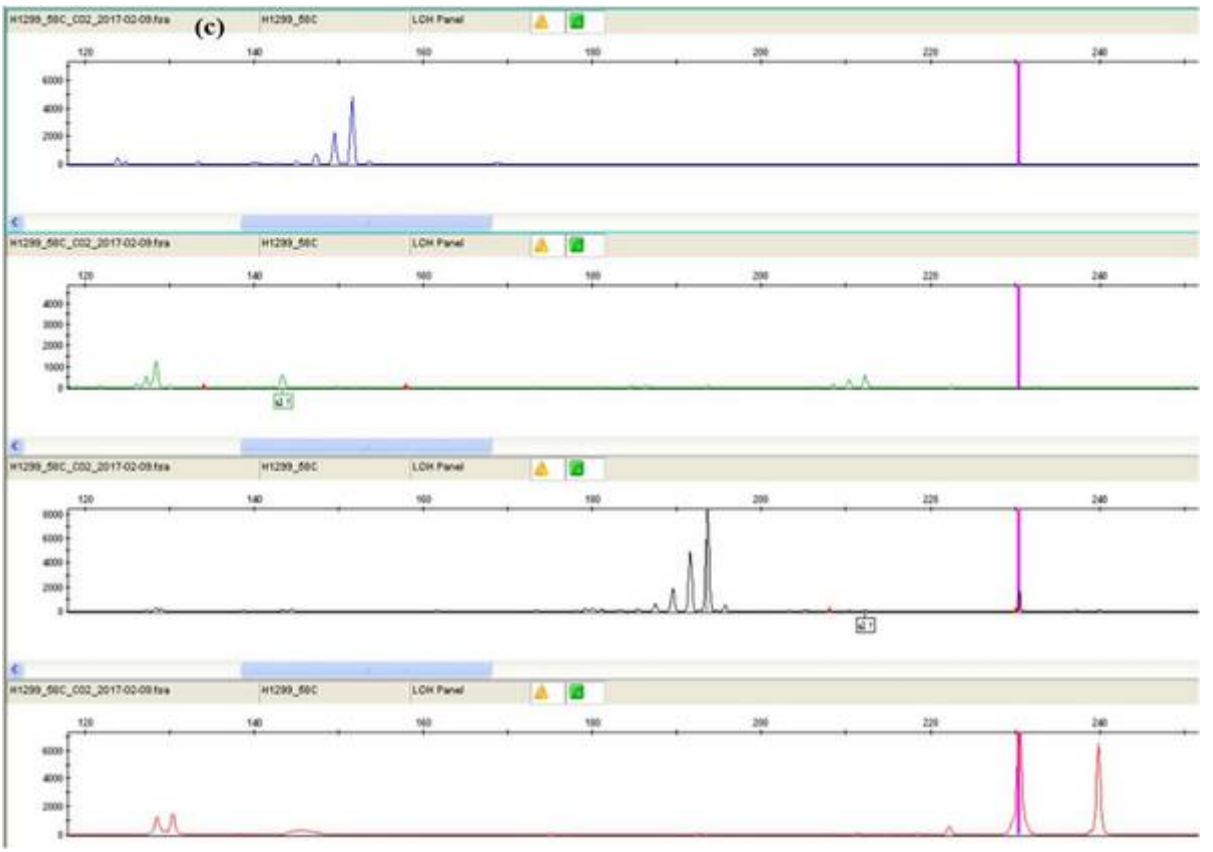
Figure 5 (a&b): Representative pictures of the microsatellites markers peaks observed in colorectal carcinomas of the patients.. Results were normal (no LOH was observed)

From the total of 207 colorectal samples, 120 (including primary tumors and metastatic tumors) were successfully amplified and screened for loss of heterozygosity (LOH) at the LKB1/STK11 gene locus as in the remaining 87 colorectal carcinomas samples we did not manage to amplify the primers of the samples because of the damaged DNA as the quality of the tissue was not good.

Four microsatellites markers, D19S565 (**labeled black**), D19S878(**labeled green**), D19S883 (**labeled red**) and D19S886 (**labeled blue**) were screened for LOH at LKB1 locus as shown in the. Surprisingly, we did not observe LOH in any of the 120 colorectal carcinomas samples. All appeared to be normal as shown in the figures 5 (a,b) above. The cell lines H460, H1299, A549, HCC827 were used as controls for our gene mapping technique as shown in figure 6 (a,b,c,d) below.




(b)
H460.bmp
Τύπος: Αρχείο
Μέγεθος: 3,75
Διαστάσεις: 12


(c)

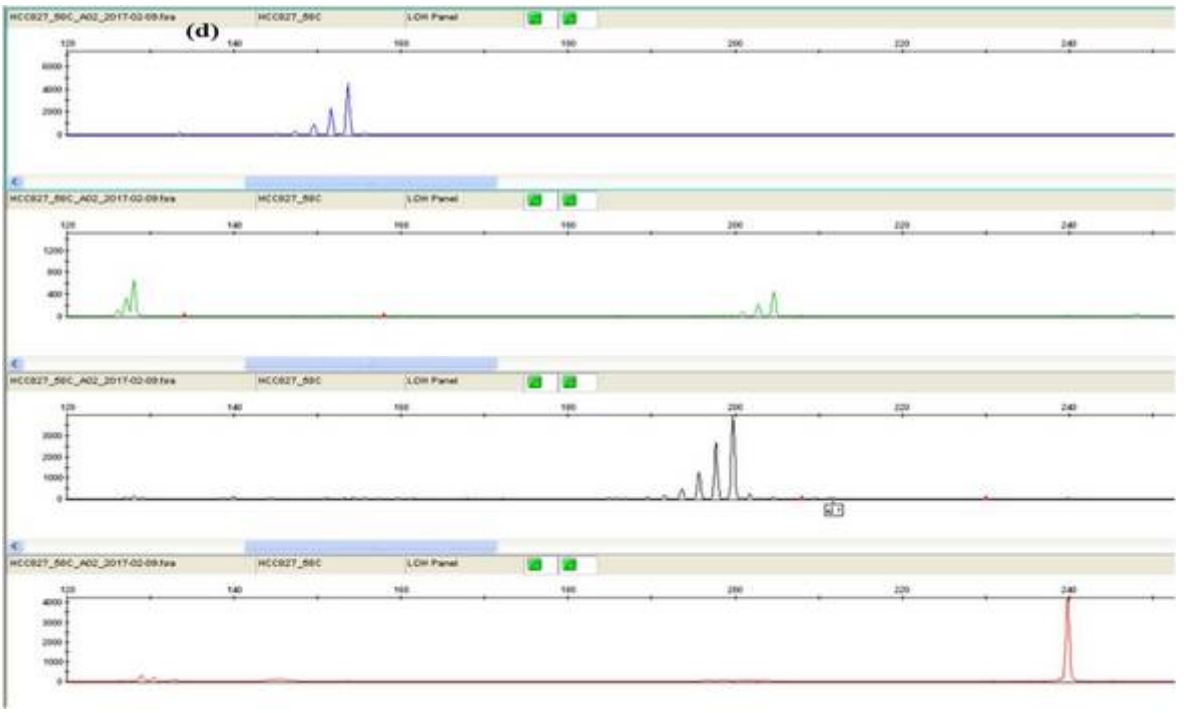


Figure 6 (a,b,c,d): Peaks of the microsatellites markers of the cell lines used as controls (Positive and negative) for LOH

4.2 Immunohistochemistry (IHC):

Immunohistochemistry was used to detect loss of heterozygosity of LKB1 by relating its expression among the patients. We have total 207 patient samples, out of which 75 were non-metastatic and 132 were metastatic. Among metastatic samples, we have eight primary metastatic tumors and 124 lymph node metastatic tumors.

4.2.1 LKB1 expression status in colorectal patients:

LKB1 expression in colorectal cancer was heterogeneous. We observed four different types of LKB1 expression i-e with no LKB1 expression at all, exclusively cytoplasmic expression, nuclear only, and both cytoplasmic and nuclear expression together. Overall 82/207 (39.6%) carcinomas exhibited negative LKB1 expression, whereas 125/207 (60.4%) carcinomas showed positive expression. Among the positive LKB1 expression patients, 96 (46.38%) of the cases have exclusively cytoplasmic expression, 27 (13.04%) patients have both cytoplasmic as well as nuclear expression simultaneously as shown graphs in figure 7 (1,2). The representative

immunohistochemical pictures are in figure 8 (a, b, c, d, e). However, only 2 (0.97%) cases (histology no. 1301-7/08 and 1862 E/05) showed exclusively nuclear expression as shown in its IHC staining in figure 9 (a,b) below.

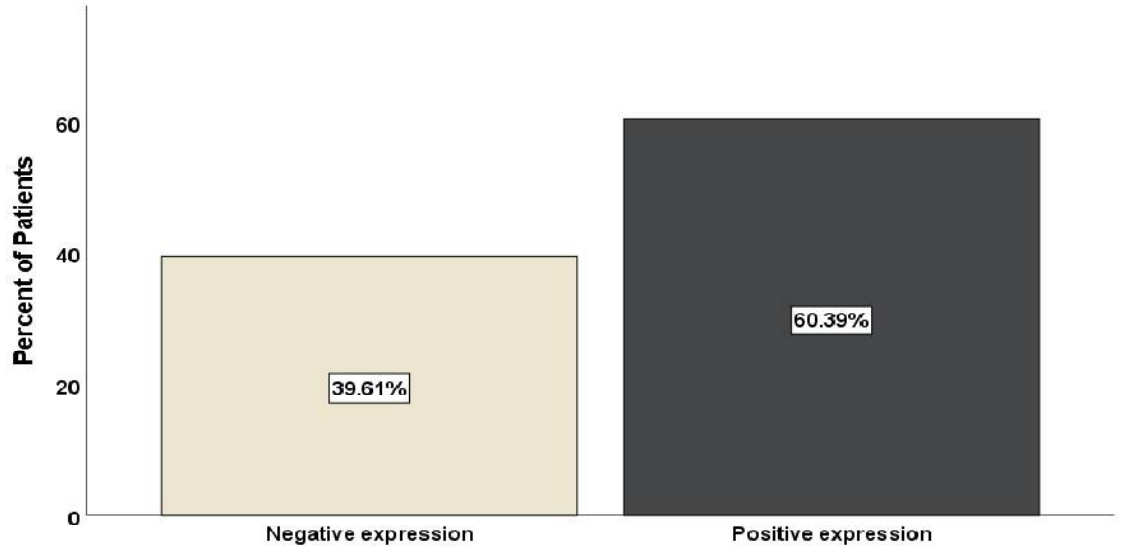


Fig: 7.1 Lkb1 Expression status in colorectal carcinomas (n=207)

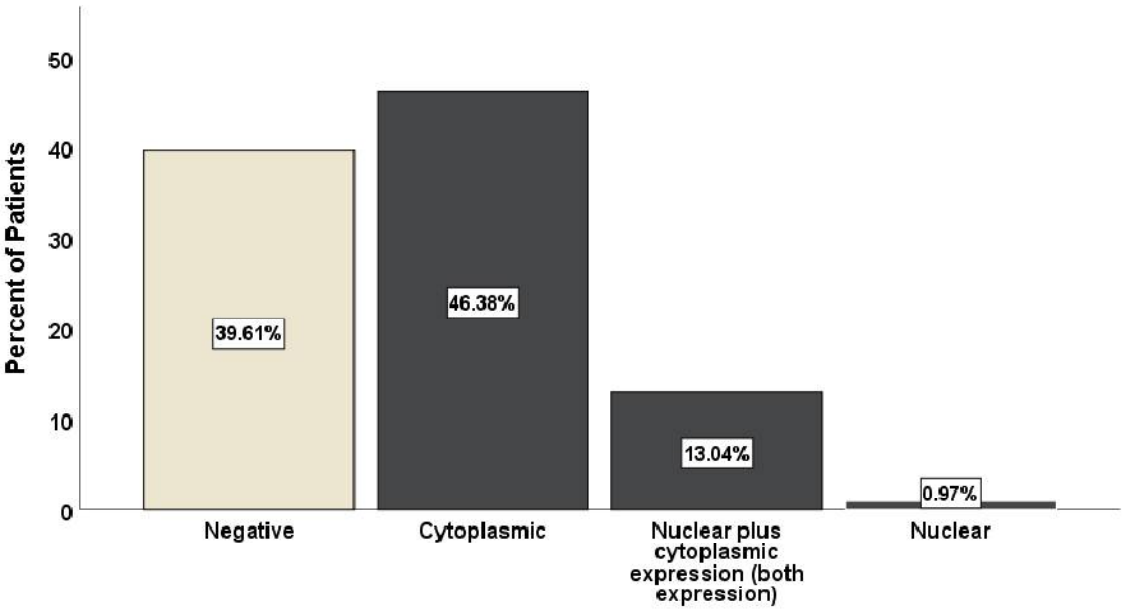


Fig: 7.2 LKB1 Expression status in colorectal carcinomas (n=207)

Figure 7 (1&2): Showing the expression status of Lkb1 in colorectal carcinomas

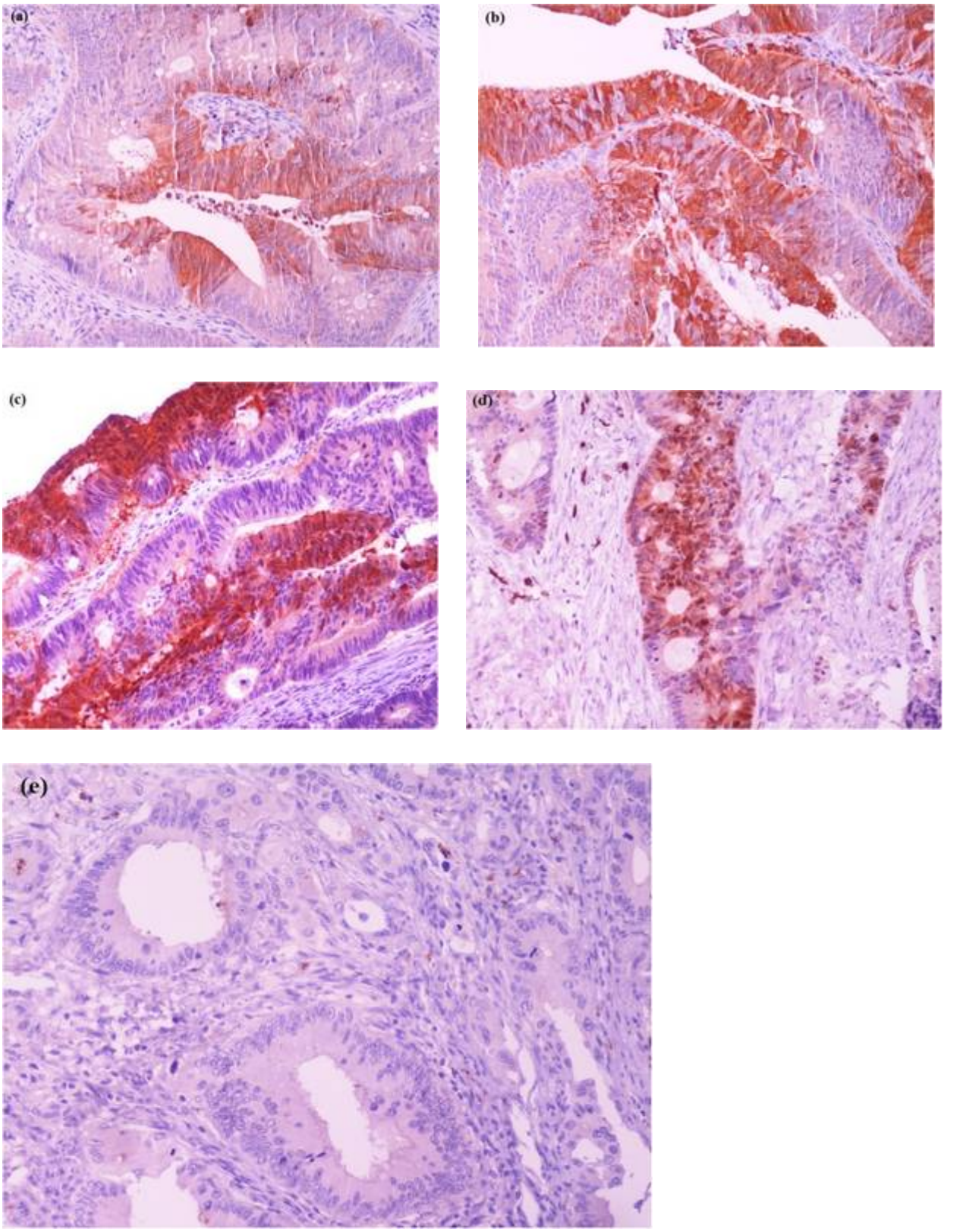


Figure 8: Showing Immunohistochemical pictures of Lkb1 expression. a & b; represent

exclusive cytoplasmic expression c &d; represent both cytoplasmic and nuclear expression together

e; represent negative expression

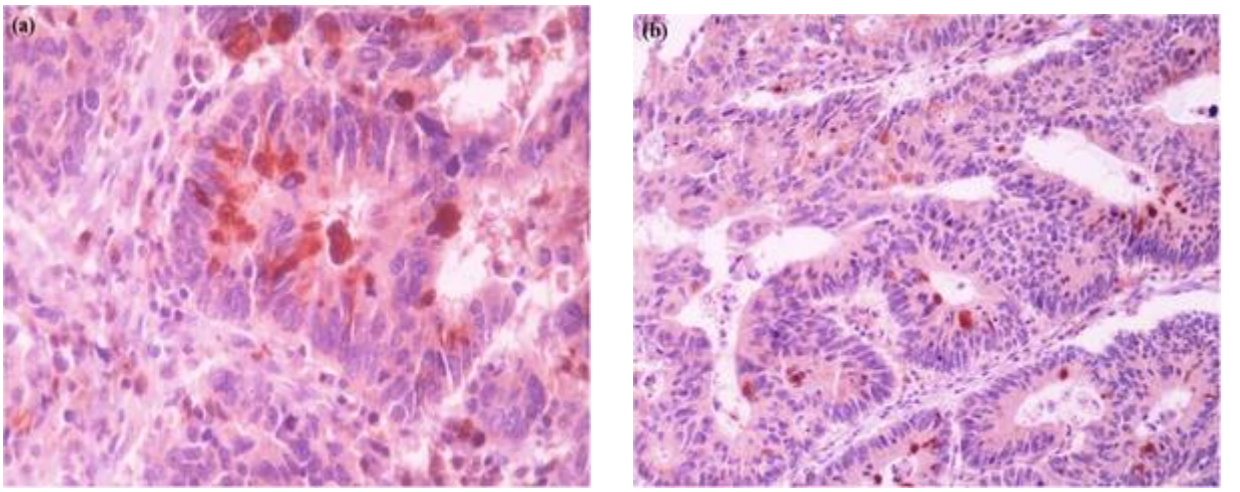


Figure 9 (a &b): Showing exclusive nuclear immunohistochemical staining of Lkb1.

In other words, we have a total 14% (29/207) cases which exhibited exclusively nuclear as well as both nuclear and cytoplasmic Lkb1 expression. The exclusively nuclear as well as simultaneously nuclear and cytoplasmic expression proposes that LKB1 inactivation has occurred possibly due to LOH in these patients because of mutant LKB1 express only nuclear expression or both nuclear and cytoplasmic expression while wild-type LKB1 express only cytoplasmic expression to carry out its normal kinase function (43).

4.2.1.1: Lkb1 expression status in metastatic colorectal carcinomas versus non-metastatic carcinomas:

We further analyzed the distribution of LKB1 expression in metastatic versus non-metastatic

patient groups. It was found that from metastatic group 62.1% (82/132) patients have positive expression while in the non-metastatic group the positive expression was observed only in 57.3% (43/75) cases. Likewise, the negative expression was observed in 37.8% metastatic patients while in a non-metastatic group it was noted only in 42.6% of cases only as shown in figure 10 (1 & 2).

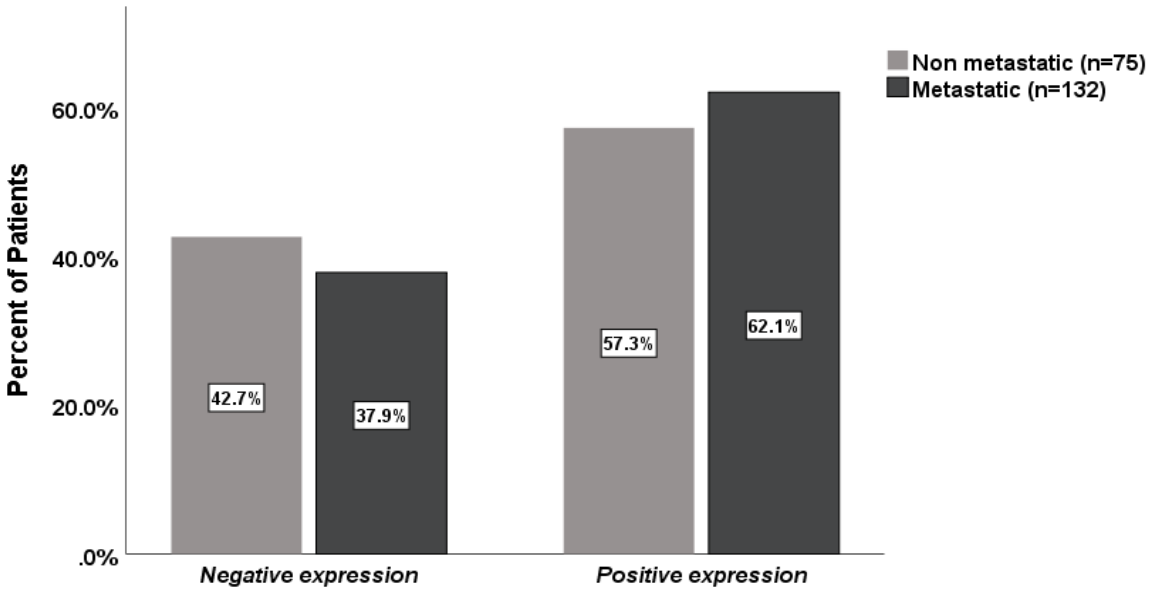


Fig: 10.1 Lkb1 Expression status in metastatic vs. non-metastatic colorectal carcinomas

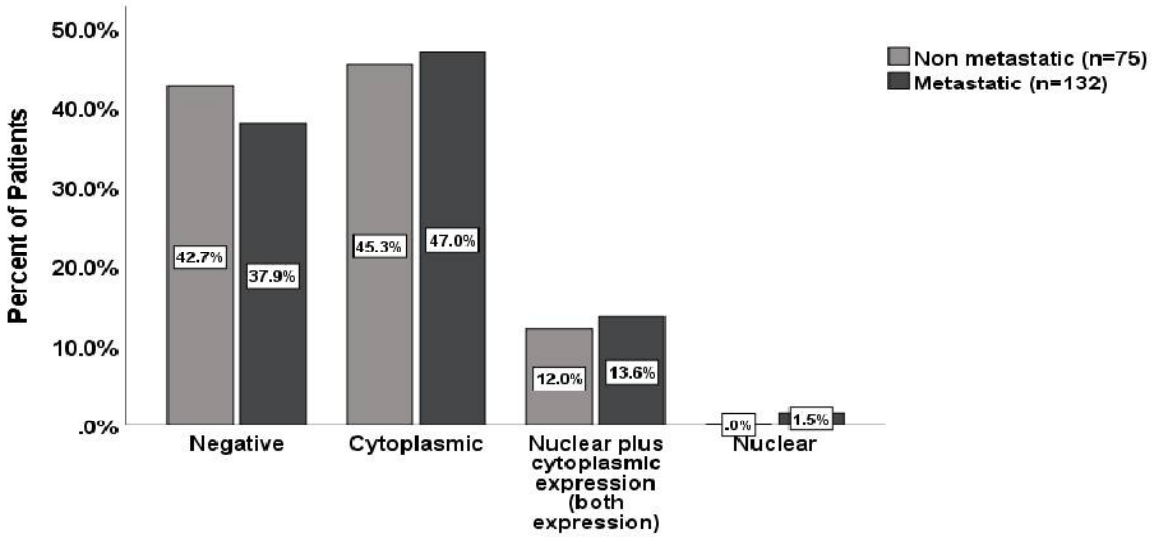


Fig: 10.2 LKB1 Expression status in metastatic vs. non-metastatic colorectal carcinomas

Figure 10: (1&2): Representing the percentage of patients in metastatic and non-metastatic

colorectal carcinomas according to their Lkb1 expression status.

In other words, metastatic patients group showed more positive expression (62.1% vs. 57.3%) than non-metastatic patients, while negative expression was higher in non-metastatic patients (42.6% vs. 37.8%) than the metastatic group, although the difference is nonsignificant. Additionally, we found that among the positive Lkb1 expression, the metastatic group has a greater number of patients (15.15% vs. 12%) than non-metastatic patients that showed both cytoplasmic as well as nuclear expression together. From the above data, we implied that metastatic colon cancer has more Lkb1 inactivation due to LOH than non-metastatic colon cancer. The exclusively nuclear as well as simultaneously nuclear and cytoplasmic expression suggests that LKB1 inactivation has occurred possibly due to LOH in these patients because of mutant LKB1 express only nuclear expression or both nuclear and cytoplasmic expression while wild-type LKB1 express only cytoplasmic expression to carry out its normal kinase function (43).

Notable, two cases (0.97%) showed exclusively nuclear staining and both were metastatic. Exclusively nuclear staining was only observed in the metastatic group only. The two nuclear cases differ from each in their overall survival and disease free interval (DFI). One patient has both shorter overall survival and DFI than the other patient (OS= 27.80 months vs. 60.20 months, DFI= 26.60 months vs. 60.20 months). Upon further analysis of the two patients, it was found that patient with shorter OS has a relapse, distant liver metastasis, and K-RAS G12D mutation while in the other patient these features were not found. K-Ras muatations occurs in about 30-50% of the colorectal cancers (163).

Besides that, we analyzed eight primary colon metastatic tumors by IHC, and the expression of LKB1 was heterogeneous as 3 out of 8 showed both nuclear and cytoplasmic expression simultaneously, 3/8 have loss of expression and 2 revealed exclusively cytoplasmic expression.

Additionally, we compared LKB1 expression distribution in lymph node (LN) positive metastatic patients group with non-metastatic patients group. It was found that LN metastatic group have an almost twice positive LKB1 expression (n= 77 vs. n=43) as compared to non-metastatic, however, there was no big difference in their LKB1 loss of expression (n=32 vs

n=47). Chi square test was applied to test if the difference is significant between the two groups and it was found to be non-significant (χ^2 = 0.443 p value= 0.606) as shown in table 1. The reason for the twice amount of positive LKB1 suggests the pro-oncogenic role of the LKB1 in colorectal metastasis instead of a tumor suppressor.

Table. 01 LKB1 Expression status in lymph node positive metastatic and non-metastatic colorectal carcinomas

Count

		metastatic LN+ & non metastatic		
		Non metastatic	Metastatic LN+	Total
Expression status in colorectal carcinomas	Negative expression	32	47	79
	Positive expression	43	77	120
Total		75	124	199

*Chi-square test p value= .606

4.2.2 LKB1 expression status in colorectal carcinomas versus paired/adjacent normal colon cells:

To evaluate whether there is a difference in LKB1 expression distribution pattern in colorectal carcinomas cells versus their paired/adjacent normal colon cells, we compared them by their IHC staining. It was found that in the patient's normal colon cells, 35.4% (37/105) cases had loss of LKB1 expression while 64.76% (68/105) cases showed positive expression as shown in figure 11.

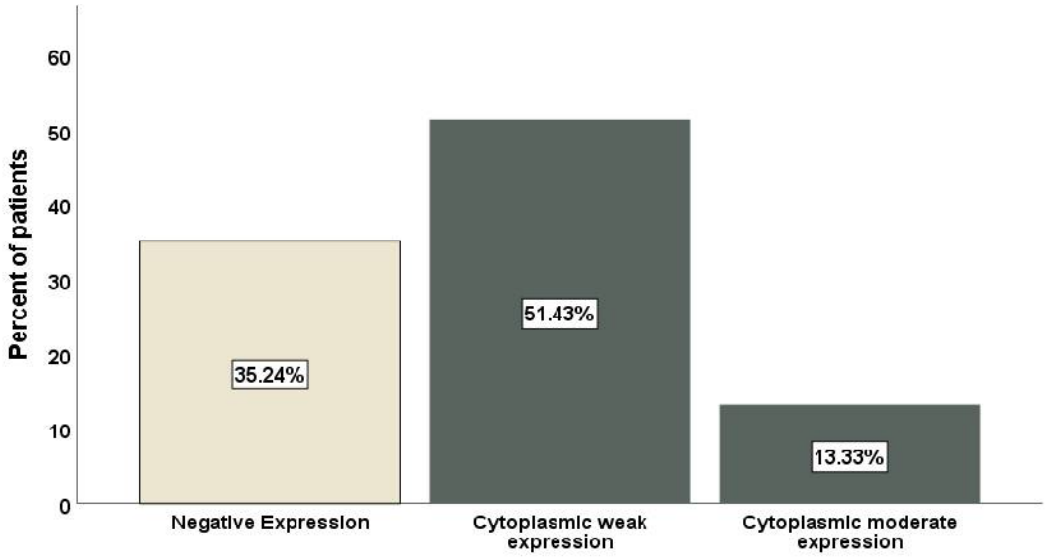


Figure: 11 LKB1 Expression in adjacent Normal colon

When the colorectal carcinomas cells were compared with their adjacent normal colon cells for LKB1 expression, it was observed that in colorectal carcinomas cells, LKB1 was expressed in two by third (66.6%) of the patients while, negative expression was found in one by third (33.3%) of the patients, suggesting that LKB1 expression distribution status was almost the same in the colorectal carcinomas as were in the paired normal colon cells. Interestingly, in half of the patients, carcinomas and their adjacent normal colon both showed positive LKB1 expression while 19% of patients showed negative Lkb1 expression in both normal colon and carcinomas. There is a strong evidence of relationship between LKB1 expression status in colorectal carcinomas and their paired normal colon cells (Chi sqaure=9.646, df=1, p= .002), although the correlation is weak (Spearman correlation=0.324, p value= 0.001) as shown in table 2.

Table: 02 LKB1 expression status in colorectal carcinomas vs. paired/adjacent normal colon (n=105)

			LKB1 Expression in Normal colon		
			Negative Expression	Positive expression	Total
LKB1 Expression status in colorectal carcinomas	Negative expression	Count	20	15	35
		% of Total	19.0%	14.3%	33.3%
	Positive expression	Count	17	53	70
		% of Total	16.2%	50.5%	66.7%
		Count	37	68	105
		% of Total	35.2%	64.8%	100.0%

*Chi-square test p-value= 0.002,
**Spearman correlation ρ=0.324 p-value= 0.001

4.2.3 Clinical Characteristics distribution according to LKB1 expression:

We examined the clinical characteristics of colorectal cancer patients according to their LKB1 expression to find out that their relationship with the LKB1 expression distribution. We first compared the clinical parameters of the patients with having an exclusive cytoplasmic expression with patients showing both cytoplasmic and nuclear expression together and then in another analysis Positive LKB1 expression versus negative expression. None of the clinical parameters was significantly associated with LKB1 expression using chi-square test except tumor T3 as shown in table 3 & 4.

Table. 03 Clinicopathologic characteristics of patients according to LKB1 IHC positive expression status only (n=123)

	No. of Patients (%)					
	Cytoplasmic Only (n=96)		**Both Cytolplasmic & Nuclear (n=27)**			**p value**
Average age y	65.22	(100)	63.67	(100)		0.481
Sex						0.777
Male	55	(57.3)	14	(51.9)		
Female	41	(42.7)	13	(48.1)		
Tumor classification						0.155
T1	–	–	–	–		
T2	7	(7.3)	5	(18.5)		
T3	86	(89.6)	22	(81.5)		
T4	3	(3.1)	–	–		
Lymph node classification						0.463
N0	36	(37.5)	12	(44.4)		
N1	41	(42.7)	8	(29.6)		
N2	19	(19.8)	7	(25.9)		
Histology mucinous						0.969
Yes	21	(21.9)	6	(22.2)		
No	75	(78.1)	21	(77.8)		
Overall Stage						0.965
II	36	(37.5)	10	(37.0)		
III	60	(62.5)	17	(63.0)		
Relapse						0.395
Yes	22	(22.9)	9	(33.3)		
No	74	(77.1)	18	(66.7)		
Metastasis status						0.841
M0	34	(35.4)	9	(33.3)		
M1	62	(64.6)	18	(66.7)		

*t-test for age
*χ^2 test for sex, tumor classification,lymph node classification & histology
*Log rank test compared OS & DFI in terms of cytoplasmic versus both cytoplasmic & nuclear expression

Table. 04 Clinicopathologic characteristics of patients according to LKB1 positive and Negative expression IHC status (n=207)

	No. of Patients (%)					
	Positive expression (n=125)		**Negative expression (n=82)**			**p value**
Average age y	64.69	(100)	63.85	(100)		0.545
Sex						0.828
Male	70	(56.0)	48	(58.5)		
Female	55	(44.0)	34	(41.5)		
Tumor classification						**0.025**
T1	–	–	2	(2.4)		
T2	12	(9.6)	10	(12.2)		
T3	110	(88.0)	62	(75.6)		
T4	3	(2.4)	8	(9.8)		
Lymph node classification						0.827
N0	48	(38.4)	35	(42.7)		
N1	51	(40.8)	31	(37.8)		
N2	26	(20.8)	16	(19.5)		
Histology mucinous						0.371
Yes	27	(21.6)	23	(28.0)		
No	98	(78.4)	59	(72.0)		
Overall Stage						0.482
II	46	(63.2)	35	(42.7)		
III	79	(36.8)	47	(57.3)		
Relapse						0.242
Yes	32	(25.6)	28	(34.1)		
No	93	(74.4)	54	(65.9)		
Metastasis status						0.597
M0	43	(34.4)	32	(39.0)		
M1	82	(65.6)	50	(61.0)		

*t-test for age
*χ^2 test for sex, tumor classification,lymph node classification & histology
*Log rank test compared OS & DFI in terms of positive versus negative expression

We further analyzed the association between LKB1 expression with overall survival (OS) and disease free interval (DFI). It was found that there was no difference in overall survivals of the patients showing exclusively cytoplasmic versus both cytoplasmic group and nuclear expression together group, however they differ in their disease free intervals (DFI), although this difference was non-significant (p value =0.104 using log-rank test) as shown in figure 12 (a,b).

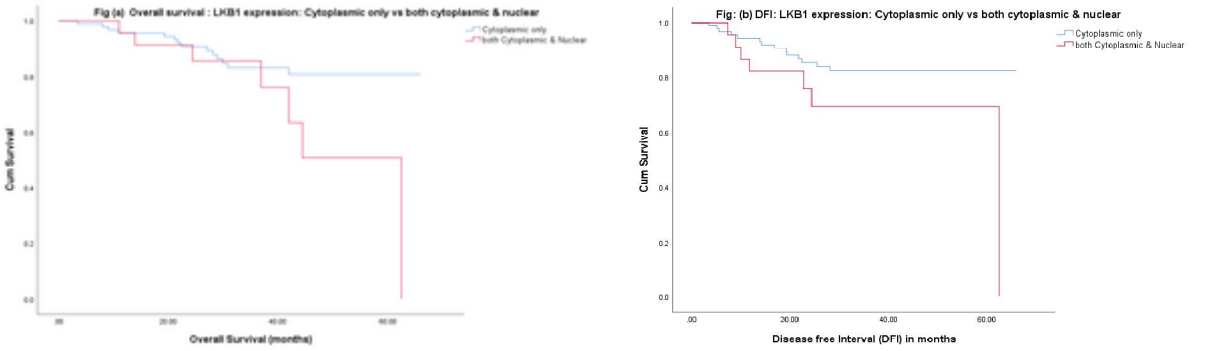


Figure 12 (a&b): Representing OS & DFI of patients having exclusively cytoplasmic expression versus both cytoplasmic and nuclear expression together. Fig a. log rank test p-value=0.102 Fig.b Log rank test p value=0.104

Similarly, OS and DFI were compared between LKB1 positive expression patients group and negative expression group. But these two group did not differ in their OS and DFI as shown in figure 13 (a,b) below. Cox proportional hazard model was used to test for OS and DFI based on the LKB1 expression groups, but the values were nonsignificant, that's why it was not included here.

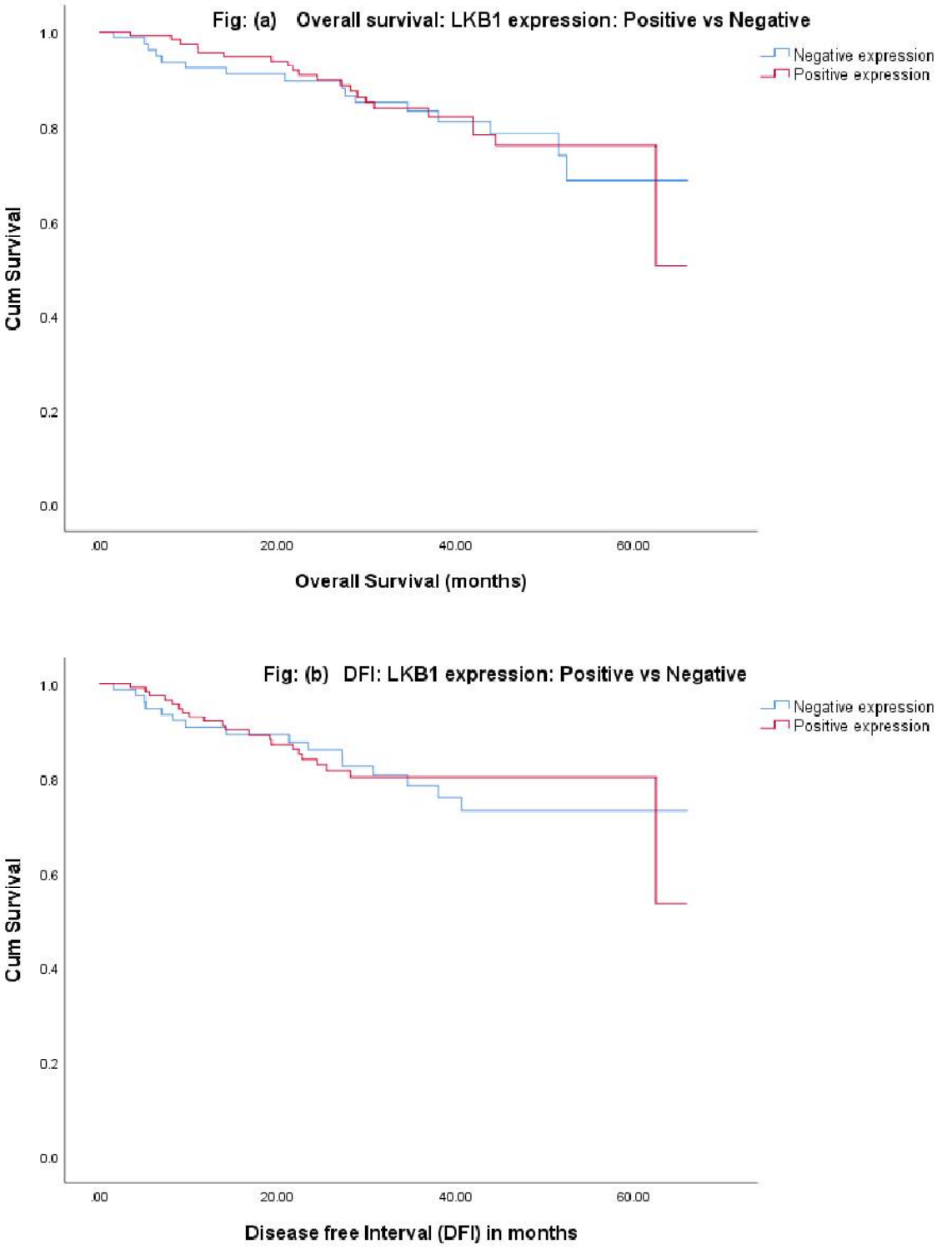


Figure 13 (a&b): Representing OS & DFI of patients having positive expression versus negative expression together. Fig a. log rank test p-value=0.886 Fig.b Log rank test p-value=0.662

5. DISCUSSION:

Different mouse models and data from human specimens have begun to provide insight into the mechanisms underlying LKB1 tumor suppressor activity. Loss of heterozygosity (LOH) of LKB1 in colorectal carcinomas has not been thoroughly studied particularly in non-metastatic and metastatic carcinomas, although there are studies about LOH of LKB1 in only limited Puetz Jegher Syndrome (PJS) patients. We tried to detect LOH in a relatively large number of samples (n=207) of colorectal carcinomas. We used two methods for the identification of LOH, I-e Gene mapping using microsatellite markers and immunohistochemistry (IHC). We did not detect LOH in any of the colorectal samples that we tested for LOH (n=120) by using gene mapping technique. Although we tried this technique for remaining samples (n=87), however, we were unable to perform it because the DNA was damaged in these samples as it could not be amplified using PCR. However, we identified LOH in 14% (29/207) samples by using IHC.

We used IHC with the end goal to recognize LOH by relating their expression to LOH. We found three predominant immunophenotypes in the samples. The expression of Lkb1 was, a: exclusively cytoplasmic expression, b: both nuclear and cytoplasmic expression, c: no expression of Lkb1. We found that from total cases, a number of 125 cases (60.4%) have positive Lkb1 expression and 82 (39.6%) cases have negative expression. Among the positive Lkb1 expression, 14% (29/207) patients showed both nuclear and cytoplasmic expression simultaneously as well as exclusive nuclear expression. Surprisingly, the two cases which showed exclusively nuclear Lkb1 staining were both from metastatic carcinomas. The exclusively nuclear as well as simultaneously nuclear and cytoplasmic expression intimates that inactivation LKB1 has happened probably due to LOH in these patients because Nezu et; al, demonstrated that mutant LKB1 express only nuclear expression or both nuclear and cytoplasmic expression while wild type LKB1 have only cytoplasmic expression to carry out its normal kinase function (43). Similarly, Tiainen et al and other authors had proposed that kinase-deficient LKB1 mutants predominantly display nuclear immunostaining and are unable to arrest G1 cell cycle arrest (61,127). Moreover, upon Akt phosphorylation, 14–3–3 protein binds to LKB1 and antagonizes the interaction with STARDα. Consequentially, LKB1 is sequestered in the nucleus as fails to inhibit cell proliferation (86).

Interestingly, in metastatic group 15.15 % (20/132) patients exhibited both exclusively nuclear and nuclear & cytoplasmic at the same time as compared to the non-metastatic group in which only 12% (9/75) showed this type of expression, which suggests that Lkb1 inactivation is more common in metastatic colorectal cancer than non-metastatic. Scott et, al illustrated that LKB1 catalytically deficient mutants activate the expression of cyclin D1 through recruitment to response elements within the promoter of the oncogene Cyclin D1, so consequently change adenomas to carcinomas (164).

Furthermore, we further analyzed each group i-e patients with metastatic and nonmetastatic colon cancer, on the basis of their Lkb1 expression. We found the metastatic group has less no of patients (37.8% vs. 42.6%) that have loss of expression than the non-metastatic group. Gene silencing due to methylation of CpG islands in the LKB1 promoter as well as biallelic inactivation of LKB1 may have caused the negative expression (loss of LKB1 expression) as suggested by Calles et, al and we have the same opinion regarding negative lKb1 expression (165). Besides that, we found that a greater number of cases from metastatic patients group (62.1% vs. 57.3%) showed positive LKB1 expression than the non-metastatic group. It can be inferred from the above discussion that loss of LKB1 may be important in initial tumor cell growth, invasion, and metastasis, perhaps re-establishment of its expression may promote tumor survival when growth outstrips nutrient. Also, the increased positive expression (62.1%) in the metastatic group suggests the pro-oncogenic role of LKb1 in metastatic colon carcinomas.

Additionally, we compared the lymph node (LN) metastatic group with non-metastatic patients, and we found greater no of patients showing positive expression in the lymph node metastatic group (n=77 vs. n=43) than the non-metastatic group. This suggests that Lkb1 might have an oncogenic role in metastatic colorectal carcinomas instead of tumor suppressor function because LKB1 pro-oncogenic activity triggers cell survival in circulating tumor cells (121). Recent research has also suggested that LKB1 can function to prevent apoptosis in times of stress, which might similarly provide tumors with a growth advantage. Under energy-deprived conditions, for example, LKB1-deficient fibroblasts were shown to undergo greater degrees of cell death than wild type counterparts (93). Similarly, it has been reported that AMPK activation by oncogenic events is required for maintaining tumor cell growth and survival in subcutaneous tumor xenografts (167), and elevated AMPK activity has been found in mouse and human brain

tumors (126,167). Likewise, Lkb1 polyubiquitination by Skp2-SCF ubiquitin ligase activates Lkb1-AMPK axis, and surprisingly oncogenic H-Ras promotes the polyubiquitination and activation of LKB1 (122). Energy stress condition especially glucose starvation induces LKB1-AMPK-mediated MMP-9 expression, which promotes cell invasiveness and migration (168). It is also shown that the LKB1/AMPK pathway protects epithelial cells from anoikis once they detach from a surface (124). Recently it was found that AMPK promotes the survival of colorectal cancer stem cells (CSCs) (123), and LKb1 is happened to be responsible as it is an upstream kinase of AMPK. Although LKB1 is classified as a tumor suppressor, its activation by Skp2 in response to oncogenic H-Ras as well as by other mechanisms suggest that it also exhibits pro-oncogenic potential and this is true in our study because there is increased activation (positively expression) of Lkb1 in a metastatic group than the non-metastatic group.

In our experiments, we identified Lkb1 inactivation possibly due to LOH in 14% (29/207) cases by using IHC; other possibilities have to be considered which can also affect Lkb1 expression. For instance, Lkb1 inactivation can occur due to methylation, somatic mutations, post-transcriptional modification as well other mechanisms for which more studies are recommended. Also, we suggest the partial role of LKb1 in colorectal cancer pathogenesis as studies of adenomas and carcinomas from patients revealed genetic alterations in other genes such as APC, PTEN, PIK3CA, BRAF, EGFR, K-ras, B-catenin, and p53 genes which play their part in the colon cancer pathogenesis (144,145,169). Furthermore, studies have shown that Lkb1 expression is significantly associated with overall survival (OS) and Disease free interval (DFI) only in the Asian population and obvious for lung cancer only (170). As expected, our results did not reveal any significant association of LKB1 expression with overall survival as our all patients were Caucasians. We found that Lkb1 expression distribution pattern was the same in both colon carcinomas and its paired normal colon (n=105) and they have a weak positive correlation (Spearman correlation=0.324, p=0.001).

Although LKB1 IHC may underestimate the true incidence of biallelic inactivation/LOH in colorectal carcinomas (as a result of tumor heterogeneity, binding of a polyclonal antibody to the truncated protein, or some other mechanism), overall, it is a reliable method for assessing LKB1 gene status. The use of IHC for LKB1 complements other methods because it covers both genetic and epigenetic mechanisms underlying loss of LKB1 function. As IHC is rapid,

inexpensive, this method may circumvent the need for more cumbersome genetic evaluation of the LKB1, particularly when sufficient quantities of tumor-rich DNA are difficult to obtain.

In summary, we employed two different techniques to identify LOH in Lkb1. We identified LOH in 14% colon cancer cases by IHC. Lkb1 expression simultaneous nuclear and cytoplasmic (catalytic mutants Lkb1) was more common in a metastatic group than the non-metastatic group. LKb1 has a pro-oncogenic role in colon cancer as opposed to its tumor suppressor role because in the metastatic group we have a higher number of patients that showed positive LKB1 expression than the non-metastatic group. Also, there is a weak correlation between normal colon and colon carcinomas on the basis of their Lkb1 expression. Even though we used gene mapping for 120 samples only, in the remaining cases (n=87), we don't have good quality samples. Likewise, Single Nucleotide Polymorphism (SNP) array can be used to detect LOH in a more accurate way for which further studies are recommended.

6. REFERENCES:

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;00:1–31.

2. Thelin C. Epidemiology of Colorectal Cancer — Incidence, Lifetime Risk Factors Statistics and Temporal Trends. 2015;(Table 1):Ch. 4. Available from: https://doi.org/10.5772/61945

3. Brenner H, Kloor M, Pox CP. Colorectal cancer. Lancet [Internet]. 2014 Apr 26 [cited 2018 Oct 26];383(9927):1490–502. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24225001

4. Vatandoust S, Price TJ, Karapetis CS. Colorectal cancer: Metastases to a single organ. World J Gastroenterol. 2015;21(41):11767–11776.

5. Punt CJA, Koopman M, Vermeulen L. From tumour heterogeneity to advances in precision treatment of colorectal cancer. Nat Rev Clin Oncol. 2017;14:235–46.

6. Pino MS, Chung DC. The Chromosomal Instability Pathway in Colon Cancer. Gastroenterology. 2010;138(6):2059–72.

7. Cancer Genom Atlas. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330–7.

8. Carethers JM, Jung BH. Genetics and Genetic Biomarkers in Sporadic Colorectal Cancer. Gastroenterology. 2015;149(5):1177–90.

9. Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer. 2009;9(7):489–99.

10. Samadder NJ, Jasperson K, Burt RW. Hereditary and Common Familial Colorectal Cancer: Evidence for Colorectal Screening. Dig Dis Sci. 2015;60(3):734–47.

11. Ma H, Brosens LAA, Offerhaus GJA, Giardiello FM, de Leng WWJ, Montgomery EA. Pathology and genetics of hereditary colorectal cancer. Pathology. 2018;50(1):49–59.

12. Jenne DE, Reimann H, Nezu J, Friedel W, Loff S, Jeschke R, et al. Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase. Nat Genet [Internet]. 1998 Jan 1 [cited 2018 Jul 28];18(1):38–43. Available from: http://www.nature.com/doifinder/10.1038/ng0198-38

13. Hemminki A, Tomlinson I, Markie D, Järvinen H, Sistonen P, Björkqvist AM, et al. Localization of a susceptibility locus for Peutz-Jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis. Nat Genet. 1997;15(1):87–90.

14. Su JY, Erikson E, Maller JL. Cloning and characterization of a novel serine/threonine protein kinase expressed in early Xenopus embryos. J Biol Chem. 1996;271(24):14430–7.

15. Trojan J, Brieger A, Raedle J, Roth WK, Zeuzem S. Peutz-Jeghers syndrome: Molecular analysis of a three-generation kindred with a novel defect in the serine threonine kinase gene STK11. Am J Gastroenterol. 1999;94(1):257–61.

16. Hezel AF, Bardeesy N. LKB1; linking cell structure and tumor suppression. Oncogene. 2008;27(55):6908–19.

17. Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, et al. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature. 1998;114(7):667–8.

18. Avizienyte E, Roth S, Loukola A, Hemminki A, Lothe RA, Stenwig AE, et al. Somatic mutations in LKB1 are rare in sporadic colorectal and testicular tumors. Cancer Res. 1998;58(10):2087–90.

19. Park WS, Moon YW, Yang YM, Kim YS, Kim YD, Fuller BG, et al. Mutations of the STK11 gene in sporadic gastric carcinoma. Int J Oncol. 1998;13(3):601–4.

20. Beggs AD, Latchford AR, Vasen HFA, Moslein G, Alonso A, Aretz S, et al. Peutz-Jeghers syndrome: a systematic review

and recommendations for management. Gut. 2010;59(7):975–86.

21. Kopacova M, Tacheci I, Rejchrt S, Bures J. Peutz-Jeghers syndrome: Diagnostic and therapeutic approach. World J Gastroenterol. 2009;15(43):5397–408.

22. Jelsig AM, Qvist N, Brusgaard K, Nielsen CB, Hansen TP, Ousager LB. Hamartomatous polyposis syndromes: A review. Orphanet J Rare Dis. 2014;9:101.

23. Chae H-D, Jeon C-H. Peutz-Jeghers syndrome with germline mutation of STK11. Ann Surg Treat Res [Internet]. 2014 Jun [cited 2018 Aug 17];86(6):325–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24949325

24. Boardman LA, Thibodeau SN, Schaid DJ, Lindor NM, McDonnell SK, Burgart LJ, et al. Increased risk for cancer in patients with the Peutz-Jeghers syndrome. Ann Intern Med. 1998;128(1):896–9.

25. Van Lier MGF, Wagner A, Mathus-Vliegen EMH, Kuipers EJ, Steyerberg EW, Van Leerdam ME. High cancer risk in peutz-jeghers syndrome: A systematic review and surveillance recommendations. Am J Gastroenterol. 2010;105(6):1258–64.

26. Avizienyte E, Loukola A, Roth S, Hemminki A, Tarkkanen M, Salovaara R, et al. LKB1 somatic mutations in sporadic tumors. Am J Pathol. 1999;154(3):677–681.

27. Sanchez-Cespedes M, Parrella P, Esteller M, Nomoto S, Trink B, Engles JM, et al. Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. Cancer Res. 2002;62(13):3659–62.

28. Bignell GR, Barfoot R, Seal S, Collins N, Warren W, Stratton MR. Low frequency of somatic mutations in the LKB1/Peutz-Jeghers syndrome gene in sporadic breast cancer. Cancer Res [Internet]. 1998 Apr 1 [cited 2018 Jul 28];58(7):1384–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9537235

29. Manuscript A, Pancreatic I, Syndromes C. Inherited pancreatic cancer syndromes. Solomon, Sheila Das, Siddhartha Brand Randall Whitcomb, David. 2012;18(6):485–91.

30. Su GH, Hruban RH, Bansal RK, Bova GS, Tang DJ, Shekher MC, et al. Germline and somatic mutations of the STK11/LKB1 Peutz-Jeghers gene in pancreatic and biliary cancers. Am J Pathol. 1999;154(6):1835–40.

31. Guldberg P, thor Straten P, Ahrenkiel V, Seremet T, Kirkin AF, Zeuthen J. Somatic mutation of the Peutz-Jeghers syndrome gene, LKB1/STK11, in malignant melanoma. Oncogene. 1999;18(9):1777–80.

32. Rowan A, Bataille V, MacKie R, Healy E, Bicknell D, Bodmer W, et al. Somatic mutations in the Peutz-Jeghers (LKB1/STKII) gene in sporadic malignant melanomas. J Invest Dermatol. 1999;112(4):509–11.

33. Qiu W, Schönleben F, Thaker HM, Goggins M, Su GH. A novel mutation of STK11/LKB1 gene leads to the loss of cell growth inhibition in head and neck squamous cell carcinoma. Oncogene. 2006;25(20):2937–42.

34. Kim CJ, Cho YG, Park JY, Kim TY, Lee JH, Kim HS, et al. Genetic analysis of the LKB1/STK11 gene in hepatocellular carcinomas. Eur J Cancer. 2004;40(1):136–41.

35. Alessi DR, Sakamoto K, Bayascas JR. LKB1-Dependent Signaling Pathways. Annu Rev Biochem [Internet]. 2006;75(1):137–63. Available from: http://www.annualreviews.org/doi/10.1146/annurev.biochem.75.103004.142702

36. Rowan A, Churchman M, Jefferey R, Hanby A, Poulsom R, Tomlinson I. In situ analysis of LKB1/STK11 mRNA expression in human normal tissues and tumours. J Pathol. 2000;192(2):203–6.

37. Towler MC, Fogarty S, Hawley S a, Pan D a, Martin DM a, Morrice N a, et al. A novel short splice variant of the tumour suppressor LKB1 is required for spermiogenesis. Biochem J. 2008;416(1):1–14.

38. Smith DP, Spicer J, Smith A, Swift S, Ashworth A. The mouse Peutz-Jeghers syndrome gene Lkb1 encodes a nuclear protein kinase. Hum Mol Genet. 1999;8(8):1479–85.

39. Watts JL, Morton DG, Bestman J, Kemphues KJ. The C. elegans par-4 gene encodes a putative serine-threonine kinase required for establishing embryonic asymmetry. Development. 2000;127(7):1467–75.

40. Martin SG, St. Johnston D. A role for Drosophila LKB1 in anterior-posterior axis formation and epithelial polarity. Nature. 2003;421(6921):379–84.

41. Collins SP, Reoma JL, Gamm DM, Uhler MD. LKB1, a novel serine/threonine protein kinase and potential tumour suppressor, is phosphorylated by cAMP-dependent protein kinase (PKA) and prenylated in vivo. Biochem J. 2000;1(345):673–80.

42. Luukko K, Ylikorkala A, Tiainen M, Mäkelä TP. Expression of LKB1 and PTEN tumor suppressor genes during mouse embryonic development. Mech Dev. 1999;83(1–2):187–90.

43. Nezu JI, Oku A, Shimane M. Loss of cytoplasmic retention ability of mutant LKB1 found in Peutz-Jeghers Syndrome patients. Biochem Biophys Res Commun. 1999;261(3):750–5.

44. Boudeau J, Baas AF, Deak M, Morrice NA, Kieloch A, Schutkowski M, et al. MO25α/β interact with STRADα/β enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. EMBO J. 2003;22(19):5102–14.

45. Baas AF, Boudeau J, Sapkota GP, Smit L, Medema R, Morrice NA, et al. Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. EMBO J. 2003;22(12):3062–72.

46. Zeqiraj E, Filippi BM, Deak M, Alessi DR, Van Aalten DMF. Structure of the LKB1-STRAD-MO25 complex reveals an allosteric mechanism of kinase activation. Science (80-). 2009;326(5960):1707–11.

47. Milburn CC, Boudeau J, Deak M, Alessi DR, Van Aalten DMF. Crystal structure of MO25α in complex with the C terminus of the pseudo kinase STE20-related adaptor. Nat Struct Mol Biol. 2004;(11):193–200.

48. Nony P, Gaude H, Rossel M, Fournier L, Rouault JP, Billaud M. Stability of the Peutz-Jeghers syndrome kinase LKB1 requires its binding to the molecular chaperones Hsp90/Cdc37. Oncogene. 2003;22(57):9165–75.

49. BOUDEAU J, DEAK M, LAWLOR MA, MORRICE NA, ALESSI DR. Heat-shock protein 90 and Cdc37 interact with LKB1 and regulate its stability. Biochem J. 2003;370(3):849–57.

50. Gaude H, Aznar N, Delay A, Bres A, Buchet-Poyau K, Caillat C, et al. Molecular chaperone complexes with antagonizing activities regulate stability and activity of the tumor suppressor LKB1. Oncogene. 2012;31(12):1582–91.

51. Karuman P, Gozani O, Odze RD, Zhou XC, Zhu H, Shaw R, et al. The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death. Mol Cell. 2001;7(6):1307–19.

52. Smith DP, Rayter SI, Niederlander C, Spicer J, Jones CM, Ashworth A. LIP1, a cytoplasmic protein functionally linked to the Peutz-Jeghers syndrome kinase LKB1. Hum Mol Genet. 2001;10(25):2869–77.

53. Mehenni H, Lin-Marq N, Buchet-Poyau K, Reymond A, Collart MA, Picard D, et al. LKB1 interacts with and phosphorylates PTEN: A functional link between two proteins involved in cancer predisposing syndromes. Hum Mol Genet. 2005;14(15):2207–19.

54. Marignani PA, Kanai F, Carpenter CL. LKB1 Associates with Brg1 and Is Necessary for Brg1-induced Growth Arrest. J Biol Chem. 2001;276(35):32415–8.

55. Blumer JB, Bernard ML, Peterson YK, Nezu JI, Chung P, Dunican DJ, et al. Interaction of activator of G-protein signaling 3 (AGS3) with LKB1, a serine/threonine kinase involved in cell polarity and cell cycle progression. Phosphorylation of the G-protein regulatory (GPR) motif as a regulatory mechanism for the interaction of GP. J Biol Chem. 2003;278(26):23217–20.

56. Clements WK, Kimelman D. Wnt signalling gets XEEKy. Nat Cell Biol. 2003;5(10):861–3.

57. Fernandes N, Sun Y, Chen S, Paul P, Shaw RJ, Cantley LC, et al. DNA damage-induced association of ATM with its target proteins requires a protein interaction domain in the N terminus of ATM. J Biol Chem. 2005;280(15):15158–64.

58. Setogawa T, Shinozaki-Yabana S, Masuda T, Matsuura K, Akiyama T. The tumor suppressor LKB1 induces p21 expression in collaboration with LMO4, GATA-6, and Ldb1. Biochem Biophys Res Commun. 2006;943(4):1186–90.

59. Zhang Y-LL, Guo H, Zhang C-SSC, Lin SYS-CCS-Y, Yin Z, Peng Y, et al. AMP as a low-energy charge signal autonomously initiates assembly of axin-ampk-lkb1 complex for AMPK activation. Cell Metab [Internet]. 2013 Oct 1 [cited 2018 Oct 25];18(4):546–55. Available from: http://dx.doi.org/10.1016/j.cmet.2013.09.005

60. Tiainen M, Ylikorkala A, Mäkelä TP. Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest. Proc Natl Acad Sci U S A. 1999;96(16):9248–51.

61. Tiainen M, Vaahtomeri K, Ylikorkala A, Mäkelä TP. Growth arrest by the LKB1 tumor suppressor: induction of p21(WAF1/CIP1). Hum Mol Genet. 2002;11(13):1497–504.

62. Zhong DS, Sun LL, Dong LX. Molecular mechanisms of LKB1 induced cell cycle arrest. Thorac Cancer. 2013;4(3):229–33.

63. Boudeau J, Kieloch A, Alessi DR, Stella A, Guanti G, Resta N. Functional analysis of LKB1/STK11 mutants and two aberrant isoforms found in Peutz-Jeghers Syndrome patients. Hum Mutat. 2003;21(2):172.

64. Qanungo S, Haldar S, Basu A. Restoration of silenced Peutz-Jeghers syndrome gene, LKB1, induces apoptosis in pancreatic carcinoma cells. Neoplasia. 2003;5(4):367–74.

65. Bouchekioua-Bouzaghou K, Poulard C, Rambaud J, Lavergne E, Hussein N, Billaud M, et al. LKB1 when associated with methylatedER?? is a marker of bad prognosis in breast cancer. Int J Cancer. 2014;15(135):1307–18.

66. Ghaffar H, Sahin F, Su GH, Zahurak M, Sidransky D, Westra WH. LKB1 Protein Expression in the Evolution of Glandular Neoplasia of the Lung LKB1 Protein Expression in the Evolution of Glandular Neoplasia of the Lung. Clin Cancer Res. 2003;9:2998–3003.

67. Sahin F, Maitra A, Argani P, Sato N, Maehara N, Montgomery E, et al. Loss of Stk11/Lkb1 expression in pancreatic and biliary neoplasms. Mod Pathol. 2003;16(7):686–91.

68. Wei C, Amos CI, Rashid A, Sabripour M, Nations L, McGarrity TJ, et al. Correlation of Staining for LKB1 and COX-2 in Hamartomatous Polyps and Carcinomas from Patients with Peutz-Jeghers Syndrome. J Histochem Cytochem. 2003;51(12):1665–72.

69. Fenton H, Carlile B, Montgomery EA, Carraway H, Herman J, Sahin F, et al. LKB1 protein expression in human breast cancer. Appl Immunohistochem Mol Morphol [Internet]. 2006;14(2):146–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16785781

70. Kline ER, Muller S, Pan L, Tighiouart M, Chen ZG, Marcus AI. Localization-specific LKB1 loss in head and neck squamous cell carcinoma metastasis. Head Neck [Internet]. 2011 Oct [cited 2018 Aug 17];33(10):1501–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21928424

71. Dorfman J, Macara IG. STRAD Regulates LKB1 Localization by Blocking Access to Importin-a, and by Association with Crm1 and Exportin-7. Mol Biol Cell. 2008;19(4):1614–26.

72. Zhang S, Schafer-Hales K, Khuri FR, Zhou W, Vertino PM, Marcus AI. The tumor suppressor LKB1 regulates lung cancer cell polarity by mediating cdc42 recruitment and activity. Cancer Res. 2008;68(3):740–8.

73. Ylikorkala A, Avizienyte E, Tomlinson IPM, Tiainen M, Roth S, Loukola A, et al. Mutations and impaired function of LKB1 in familial and non-familial Peutz-Jeghers syndrome and a sporadic testicular cancer. Hum Mol Genet. 1999;8(1):45–51.

74. Forcet C, Etienne-Manneville S, Gaude H, Fournier L, Debilly S, Salmi M, et al. Functional analysis of Peutz-Jeghers mutations reveals that the LKB1 C-terminal region exerts a crucial role in regulating both the AMPK pathway and the cell polarity. Hum Mol Genet. 2005;14(10):1283–92.

75. Dogliotti G, Kullmann L, Dhumale P, Thiele C, Panichkina O, Mendl G, et al. Membrane-binding and activation of LKB1 by phosphatidic acid is essential for development and tumour suppression. Nat Commun. 2017;8(26):15747-.

76. Li W, Wong CC, Zhang X, Kang W, Nakatsu G, Zhao Q, et al. CAB39L elicited an anti-Warburg effect via a LKB1-AMPK-PGC1α axis to inhibit gastric tumorigenesis. Oncogene [Internet]. 2018; Available from: http://dx.doi.org/10.1038/s41388-018-0402-1

77. Esteller M, Avizienyte E, Corn PG, Lothe R a, Baylin SB, Aaltonen L a, et al. Epigenetic inactivation of LKB1 in primary tumors associated with the Peutz-Jeghers syndrome. Oncogene. 2000;19(1):164–8.

78. Trojan J, Brieger A, Raedle J, Esteller M, Zeuzem S, Ii MK, et al. Promoter and Allelic Loss At Chromosome 19P13 . 3 in Sporadic Colorectal Cancer. 2000;272–6.

79. Brown KA, McInnes KJ, Takagi K, Ono K, Hunger NI, Wang L, et al. LKB1 expression is inhibited by estradiol-17β in MCF-7 cells. J Steroid Biochem Mol Biol. 2011;127(3–5):439–43.

80. McInnes KJ, Brown KA, Hunger NI, Simpson ER. Regulation of LKB1 expression by sex hormones in adipocytes. Int J Obes. 2012;36(7):982–5.

81. Han S, Khuri FR, Roman J. Fibronectin stimulates non-small cell lung carcinoma cell growth through activation of Akt/mammalian target of rapamycin/S6 kinase and inactivation of LKB1/AMP-activated protein kinase signal pathways. Cancer Res. 2006;66(1):315–23.

82. Kullmann L, Krahn MP. Controlling the master—upstream regulation of the tumor suppressor LKB1. Oncogene [Internet]. 2018;1–13. Available from: http://dx.doi.org/10.1038/s41388-018-0145-z

83. Xie Z, Dong Y, Zhang J, Scholz R, Neumann D, Zou M-H. Identification of the Serine 307 of LKB1 as a Novel Phosphorylation Site Essential for Its Nucleocytoplasmic Transport and Endothelial Cell Angiogenesis. Mol Cell Biol [Internet]. 2009;29(13):3582–96. Available from: http://mcb.asm.org/cgi/doi/10.1128/MCB.01417-08

84. SAPKOTA GP, BOUDEAU J, DEAK M, KIELOCH A, MORRICE N, ALESSI DR. Identification and characterization of four novel phosphorylation sites (Ser31, Ser325, Thr336 and Thr366) on LKB1/STK11, the protein kinase mutated in Peutz–Jeghers cancer syndrome. Biochem J. 2002;

85. Luo L, Huang W, Tao R, Hu N, Xiao ZX, Luo Z. ATM and LKB1 dependent activation of AMPK sensitizes cancer cells to etoposide-induced apoptosis. Cancer Lett. 2013;

86. Liu L, Siu FM, Che CM, Xu A, Wang Y. Akt blocks the tumor suppressor activity of LKB1 by promoting phosphorylation-dependent nuclear retention through 14-3-3 proteins. Am J Transl Res. 2012;4(2):175–8.

87. Xie Z, Dong Y, Scholz R, Neumann D, Zou MH. Phosphorylation of LKB1 at serine 428 by protein kinase C-?? is required for metformin-enhanced activation of the AMP-activated protein kinase in endothelial cells. Circulation. 2008;

88. Zhu H, Moriasi CM, Zhang M, Zhao Y, Zou MH. Phosphorylation of serine 399 in LKB1 protein short form by protein kinase C?? is required for its nucleocytoplasmic transport and consequent AMP-activated protein kinase (AMPK) activation. J Biol Chem. 2013;288(23):16495–505.

89. Sapkota GP, Deak M, Kieloch A, Morrice N, Goodarzi AA, Smythe C, et al. Ionizing radiation induces ataxia telangiectasia mutated kinase (ATM)-mediated phosphorylation of LKB1/STK11 at Thr-366. Biochem J [Internet]. 2002 Dec 1 [cited 2018 Oct 25];368(Pt 2):507–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12234250

90. Zu Y, Liu L, Lee MYK, Xu C, Liang Y, Man RY, et al. SIRT1 promotes proliferation and prevents senescence through targeting LKB1 in primary porcine aortic endothelial cells. Circ Res. 2010;

91. Lan F, Cacicedo JM, Ruderman N, Ido Y. SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1: Possible role in AMP-activated protein kinase activation. J Biol Chem. 2008;283(41):27628–35.

92. Lizcano JM, Göransson O, Toth R, Deak M, Morrice NA, Boudeau J, et al. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J. 2004;23(4):833–43.

93. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci [Internet]. 2004 Mar 9 [cited 2018 Oct 16];101(10):3329–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14985505

94. Carling D. The AMP-activated protein kinase cascade - A unifying system for energy control. Trends Biochem Sci. 2004;29(1):18–24.

95. Shaw RJ. Glucose metabolism and cancer. Curr Opin Cell Biol. 2006;18(6):598–606.

96. Hardie DG. AMP-activated/SNF1 protein kinases: Conserved guardians of cellular energy. Nat Rev Mol Cell Biol. 2007;8(10):774–85.

97. Corradetti MN, Inoki K, Bardeesy N, DePinho RA, Guan KL. Regulation of the TSC pathway by LKB1: Evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev. 2004;18(13):1533–8.

98. Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell. 2004;6(1):91–9.

99. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. Mol Cell. 2008;30(2):214–6.

100. Xie Z, Dong Y, Zhang M, Cui M-Z, Cohen R a, Riek U, et al. Activation of protein kinase C zeta by peroxynitrite regulates LKB1-dependent AMP-activated protein kinase in cultured endothelial cells. J Biol Chem. 2006;283(10):6366–75.

101. Song P, Xie Z, Wu Y, Xu J, Dong Y, Zou MH. Protein kinase C??-dependent LKB1 serine 428 phosphorylation increases LKB1 nucleus export and apoptosis in endothelial cells. J Biol Chem. 2008;283(18):12446–55.

102. Sakamoto K, McCarthy A, Smith D, Green KA, Hardie DG, Ashworth A, et al. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. EMBO J. 2005;24(10):1810–20.

103. Koh H-J, Arnolds DE, Fujii N, Tran TT, Rogers MJ, Jessen N, et al. Skeletal Muscle-Selective Knockout of LKB1 Increases Insulin Sensitivity, Improves Glucose Homeostasis, and Decreases TRB3. Mol Cell Biol. 2006;26(22):8217–8227.

104. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, DePinho RA, et al. Medicine: The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science (80-). 2005;310(5754):1642–6.

105. Gan RY, Li H Bin. Recent progress on liver kinase B1 (LKB1): Expression, regulation, downstream signaling and cancer suppressive function. Int J Mol Sci. 2014;15(9):16698–718.

106. Rossi DJ, Ylikorkala A, Korsisaari N, Salovaara R, Luukko K, Launonen V, et al. Induction of cyclooxygenase-2 in a mouse model of Peutz-Jeghers polyposis. Proc Natl Acad Sci. 2002;99(19):12327–32.

107. Partanen JI, Nieminen AI, Makela TP, Klefstrom J. Suppression of oncogenic properties of c-Myc by LKB1-controlled epithelial organization. Proc Natl Acad Sci. 2007;104(37):14694–14699.

108. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, et al. The energy sensing LKB1-AMPK pathway regulates p27kip1phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol. 2007;9(2):218–24.

109. Harper JW, Elledge SJ. The DNA Damage Response: Ten Years After. Mol Cell. 2007;14(28):739–45.

110. Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, Hurov KE, Luo J, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science (80-). 2007;316(5828):1160–6.

111. Zhou W, Zhang J, Marcus AI. LKB1 tumor suppressor: Therapeutic opportunities knock when LKB1 is inactivated. Genes Dis. 2014;1(1):64–74.

112. Ui A, Ogiwara H, Nakajima S, Kanno S, Watanabe R, Harata M, et al. Possible involvement of LKB1-AMPK signaling in non-homologous end joining. Oncogene. 2014;33(13):1640–8.

113. Ji H, Ramsey MR, Hayes DN, Fan C, McNamara K, Kozlowski P, et al. LKB1 modulates lung cancer differentiation and metastasis. Nature. 2007;448(7155):807–10.

114. Liu W, Monahan KB, Pfefferle AD, Shimamura T, Sorrentino J, Chan KT, et al. LKB1/STK11 Inactivation Leads to Expansion of a Prometastatic Tumor Subpopulation in Melanoma. Cancer Cell. 2012;21(6):751–64.

115. Shorning BY, Griffiths D, Clarke AR. Lkb1 and Pten synergise to suppress mTOR-Mediated tumorigenesis and epithelial-mesenchymal transition in the mouse bladder. PLoS One. 2011;6(1):e16209.

116. Roy BC, Kohno T, Iwakawa R, Moriguchi T, Kiyono T, Morishita K, et al. Involvement of LKB1 in epithelial-mesenchymal transition (EMT) of human lung cancer cells. Lung Cancer. 2010;70(2):136–45.

117. Cheng H, Liu P, Wang ZC, Zou L, Santiago S, Garbitt V, et al. SIK1 couples LKB1 to p53-dependent anoikis and suppresses metastasis. Sci Signal. 2009;2(80):ra35.

118. Wingo SN, Gallardo TD, Akbay EA, Liang MC, Contreras CM, Boren T, et al. Somatic LKB1 mutations promote cervical cancer progression. PLoS One. 2009;4(4):e5137.

119. Contreras CM, Gurumurthy S, Haynie JM, Shirley LJ, Akbay EA, Wingo SN, et al. Loss of Lkb1 provokes highly invasive endometrial adenocarcinomas. Cancer Res. 2008;68(3):759–66.

120. Contreras CM, Akbay EA, Gallardo TD, Haynie JM, Sharma S, Tagao O, et al. Lkb1 inactivation is sufficient to drive endometrial cancers that are aggressive yet highly responsive to mTOR inhibitor monotherapy. Dis Model Mech. 2010;3(3–4):181–93.

121. Trapp EK, Majunke L, Zill B, Sommer H, Andergassen U, Koch J, et al. LKB1 pro-oncogenic activity triggers cell survival in circulating tumor cells. Mol Oncol. 2017;11(11):1508–26.

122. Lee SW, Li CF, Jin G, Cai Z, Han F, Chan CH, et al. Skp2-Dependent Ubiquitination and Activation of LKB1 Is Essential for Cancer Cell Survival under Energy Stress. Mol Cell. 2015;57(6):1022–33.

123. Guo B, Han X, Tkach D, Dong SH. AMPK promotes the survival of colorectal cancer stem cells. 2018;1(2):134–42.

124. Ng TL, Leprivier G, Robertson MD, Chow C, Martin MJ, Laderoute KR, et al. The AMPK stress response pathway mediates anoikis resistance through inhibition of mTOR and suppression of protein synthesis. Cell Death Differ [Internet]. 2012 Mar 23 [cited 2018 Oct 25];19(3):501–10. Available from: http://www.nature.com/articles/cdd2011119

125. Peart T, Valdes YR, Correa RJM, Fazio E, Bertrand M, McGee J, et al. Intact LKB1 activity is required for survival of dormant ovarian cancer spheroids. Oncotarget [Internet]. 2015 Sep 8 [cited 2018 Oct 25];6(26):22424–38. Available from: http://www.oncotarget.com/fulltext/4211

126. Rios M, Foretz M, Viollet B, Prieto A, Fraga M, Costoya JA, et al. AMPK Activation by Oncogenesis Is Required to Maintain Cancer Cell Proliferation in Astrocytic Tumors. Cancer Res [Internet]. 2013 Apr 15 [cited 2018 Oct 16];73(8):2628–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23370326

127. Vaahtomeri K, Mäkelä TP. Molecular mechanisms of tumor suppression by LKB1. FEBS Lett [Internet]. 2011;585(7):944–51. Available from: http://dx.doi.org/10.1016/j.febslet.2010.12.034

128. Chou CC, Lee KH, Lai IL, Wang D, Mo X, Kulp SK, et al. AMPK reverses the mesenchymal phenotype of cancer cells by targeting the Akt-MDM2-Foxo3a signaling axis. Cancer Res. 2014;74(17):4783–95.

129. Petursson F, Husa M, June R, Lotz M, Terkeltaub R, Liu-Bryan R. Linked decreases in liver kinase B1 and AMP-activated protein kinase activity modulate matrix catabolic responses to biomechanical injury in chondrocytes. Arthritis Res Ther. 2013;15(4):R77.

130. Ramnanan CJ, McMullen DC, Groom AG, Storey KB. The regulation of AMPK signaling in a natural state of profound metabolic rate depression. Mol Cell Biochem. 2010;335(1–2):91–105.

131. Zhuang Z-G, Di G-H, Shen Z-Z, Ding J, Shao Z-M. Enhanced Expression of LKB1 in Breast Cancer Cells Attenuates Angiogenesis, Invasion, and Metastatic Potential. Mol Cancer Res. 2006;

132. Lee JH, Kim JH, Kim JS, Chang JW, Kim SB, Park JS, et al. AMP-activated protein kinase inhibits TGF-β-, angiotensin II-, aldosterone-, high glucose-, and albumin-induced epithelial-mesenchymal transition. Am J Physiol Physiol. 2013;

133. Hearle NCM. Exonic STK11 deletions are not a rare cause of Peutz-Jeghers syndrome. J Med Genet. 2005;

134. Volikos E, Robinson J, Aittomäki K, Mecklin JP, Järvinen H, Westerman AM, et al. LKB1 exonic and whole gene deletions are a common cause of Peutz-Jeghers syndrome. J Med Genet. 2006;43(5):e18.

135. Giardiello FM, Brensinger JD, Tersmette AC, Goodman SN, Petersen GM, Booker S V, et al. Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology. 2000;119(6):1447–53.

136. Hearle N, Schumacher V, Menko FH, Olschwang S, Boardman LA, Gille JJP, et al. Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. Clin Cancer Res. 2006;

137. Mehenni H, Gehrig C, Nezu J, Oku A, Shimane M, Rossier C, et al. Loss of LKB1 Kinase Activity in Peutz-Jeghers Syndrome, and Evidence for Allelic and Locus Heterogeneity. Am J Hum Genet. 1998;63(3):1641–50.

138. Daniell J, Plazzer J-P, Perera A, Macrae F. An exploration of genotype-phenotype link between Peutz-Jeghers syndrome and STK11: a review. Fam Cancer [Internet]. 2018 Jul 12 [cited 2018 Aug 17];17(3):421–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28900777

139. Koivunen JP, Kim J, Lee J, Rogers AM, Park JO, Zhao X, et al. Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients. Br J Cancer. 2008;99(2):245–52.

140. Sanchez-Cespedes M. A role for LKB1 gene in human cancer beyond the Peutz-Jeghers syndrome. Oncogene. 2007;26(57):7825–32.

141. Matsumoto S, Iwakawa R, Takahashi K, Kohno T, Nakanishi Y, Matsuno Y, et al. Prevalence and specificity of LKB1 genetic alterations in lung cancers. Oncogene. 2007;

142. Trojan J, Brieger A, Raedle J, Esteller M, Zeuzem S. 5 ’-CpG island methylation of the LKB1/STK11 promoter and allelic loss at chromosome 19p13.3 in sporadic colorectal cancer. Gut. 2000;47(2):272–6.

143. Gorringe KL. Loss of Heterozygosity. In: eLS [Internet]. Chichester, UK: John Wiley & Sons, Ltd; 2016 [cited 2018 Aug 9]. p. 1–8. Available from: http://doi.wiley.com/10.1002/9780470015902.a0026643

144. Entius MM, Keller JJ, Westerman AM, Van Rees BP, Van Velthuysen MLF, De Goeij AFPM, et al. Molecular genetic alterations in hamartomatous polyps and carcinomas of patients with Peutz-Jeghers syndrome. J Clin Pathol. 2001;

145. Miyaki M, Iijima T, Hosono K, Ishii R, Yasuno M, Mori T, et al. Somatic mutations of LKB1 and β-catenin genes in gastrointestinal polyps from patients with Peutz-Jeghers syndrome. Cancer Res. 2000;

146. Gill RK, Yang SH, Meerzaman D, Mechanic LE, Bowman ED, Jeon HS, et al. Frequent homozygous deletion of the LKB1/STK11 gene in non-small cell lung cancer. Oncogene. 2011;30(35):3784–3791.

147. Fang R, Zheng C, Sun Y, Han X, Gao B, Li C, et al. Integrative genomic analysis reveals a high frequency of LKB1 genetic alteration in Chinese lung adenocarcinomas. J Thorac Oncol. 2014;

148. Wang ZJ, Ellis I, Zauber P, Iwama T, Marchese C, Talbot I, et al. Allelic imbalance at the LKB1 (STK11) locus in tumours from patients with Peutz-Jeghers’ syndrome provides evidence for a hamartoma-(adenoma)- carcinoma sequence. J Pathol. 1999;188(1):9–13.

149. Bardeesy N, Sinha M, Hezel AF, Signoretti S, Hathaway NA, Sharpless NE, et al. Loss of the Lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. Nature. 2002;

150. Miyoshi H, Nakau M, Ishikawa T, Seldin MF, Oshima M, Taketo MM. Gastrointestinal hamartomatous polyposis in Lkb1 heterozygous knockout mice. Cancer Res. 2002;62(8):2261–6.

151. Jishage K, Nezu J, Kawase Y, Iwata T, Watanabe M, Miyoshi A, et al. Role of Lkb1, the causative gene of Peutz-Jegher’s syndrome, in embryogenesis and polyposis. Proc Natl Acad Sci U S A. 2002;99(13):8903–8.

152. Morton JP, Jamieson NB, Karim SA, Athineos D, Ridgway RA, Nixon C, et al. LKB1 haploinsufficiency cooperates with Kras to promote pancreatic cancer through suppression of p21-dependent growth arrest. Gastroenterology. 2010;139(2):586–97.

153. Takeda H, Miyoshi H, Kojima Y, Oshima M, Taketo MM. Accelerated onsets of gastric hamartomas and hepatic adenomas/carcinomas in Lkb1+/-p53-/-compound mutant mice. Oncogene. 2006;25(12):1816–20.

154. Wei C, Amos CI, Stephens LC, Campos I, Deng JM, Behringer RR, et al. Mutation of Lkb1 and p53 genes exert a cooperative effect on tumorigenesis. Cancer Res. 2005;65(24):11297–303.

155. Huang X, Wullschleger S, Shpiro N, McGuire VA, Sakamoto K, Woods YL, et al. Important role of the LKB1–AMPK pathway in suppressing tumorigenesis in PTEN-deficient mice. Biochem J. 2008;412(2):211–21.

156. Katajisto P, Vaahtomeri K, Ekman N, Ventelä E, Ristimäki A, Bardeesy N, et al. LKB1 signaling in mesenchymal cells required for suppression of gastrointestinal polyposis. Nat Genet. 2008;40(4):455–9.

157. Sobottka SB, Haase M, Fitze G, Hahn M, Schackert HK, Schackert G. Frequent loss of heterozygosity at the 19p13.3 locus without LKB1/STK11 mutations in human carcinoma metastases to the brain. J Neurooncol. 2000;49(3):187–95.

158. Carretero J, Medina PP, Pio R, Montuenga LM, Sanchez-Cespedes M. Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. Oncogene. 2004;

159. Grundner-Culemann K, Dybowski JN, Klammer M, Tebbe A, Schaab C, Daub H. Comparative proteome analysis across non-small cell lung cancer cell lines. J Proteomics. 2015;

160. Cowley GS, Weir BA, Vazquez F, Tamayo P, Scott JA, Rusin S, et al. Parallel genome-scale loss of function screens in 216 cancer cell lines for the identification of context-specific genetic dependencies. Sci Data. 2014;(1):140035.

161. Conde E, Suarez-Gauthier A, García-García E, Lopez-Rios F, Lopez-Encuentra A, García-Lujan R, et al. Specific pattern of LKB1 and phospho-acetyl-CoA carboxylase protein immunostaining in human normal tissues and lung carcinomas. Hum Pathol. 2007;38(9):1351–60.

162. Zhang X, Hunt JL, Landsittel DP, Muller S, Adler-Storthz K, Ferris RL, et al. Correlation of protease-activated receptor-1 with differentiation markers in squamous cell carcinoma of the head and neck and its implication in lymph node metastasis. Clin Cancer Res [Internet]. 2004 Dec 15 [cited 2018 Aug 27];10(24):8451–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15625067

163. Tan C, Du X. KRAS mutation testing in metastatic colorectal cancer. World J Gastroenterol. 2012;18(37):5171–80.

164. Scott KD, Nath-Sain S, Agnew MD, Marignani PA. LKB1 catalytically deficient mutants enhance cyclin D1 expression. Cancer Res. 2007;67(12):5622-.

165. Calles A, Sholl LM, Rodig SJ, Pelton AK, Hornick JL, Butaney M, et al. Immunohistochemical Loss of LKB1 Is a biomarker for more aggressive biology inKRAS-mutant lung Adenocarcinoma. Clin Cancer Res. 2015;21(12):2851–60.

166. Laderoute KR, Amin K, Calaoagan JM, Knapp M, Le T, Orduna J, et al. 5'-AMP-Activated Protein Kinase (AMPK) Is Induced by Low-Oxygen and Glucose Deprivation Conditions Found in Solid-Tumor Microenvironments. Mol Cell Biol [Internet]. 2006 Jul 15 [cited 2018 Oct 16];26(14):5336–47. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16809770

167. Jang T, Calaoagan JM, Kwon E, Samuelsson S, Recht L, Laderoute KR. 5'-AMP-activated protein kinase activity is elevated early during primary brain tumor development in the rat. Int J Cancer [Internet]. 2011 May 1 [cited 2018 Oct 16];128(9):2230–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20635388

168. Endo H, Owada S, Inagaki Y, Shida Y, Tatemichi M. Glucose starvation induces LKB1-AMPK-mediated MMP-9 expression in cancer cells. Sci Rep [Internet]. 2018 Dec 4 [cited 2018 Oct 25];8(1):10122. Available from: http://www.nature.com/articles/s41598-018-28074-w

169. Salem ME, Weinberg BA, Xiu J, El-Deiry WS, Hwang JJ, Gatalica Z, et al. Comparative molecular analyses of left-sided colon, right-sided colon, and rectal cancers. Oncotarget. 2017;8(49):86356–86368.

170. Xiao J, Zou Y, Chen X, Gao Y, Xie M, Lu X, et al. The Prognostic Value of Decreased LKB1 in Solid Tumors: A Meta-Analysis. PLoS One. 2016;11(4):1–14.