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Molecular abnormalities and cellular signaling pathways alterations in lung cancer

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Abstract

Lung cancer is the main cause of death worldwide despite the progress in surgical treatment, radiotherapy, chemotherapy and targeted therapy. It is accepted that the pathogenesis of cancer involves the accumulation of multiple molecular abnormalities over time that lead to acquired cell capabilities that can be categorized into the following functional groups: self-sufficiency in growth signals due to mutations in proto-oncogenes, insensitivity to anti-growth signals as a result of mutations affecting the tumor suppressor genes, inflammatory micro-environment, evading apoptosis due to up-regulation of anti-apoptotic or down-regulation of pre-apoptotic molecules, limitless replicative potential due to telomerase activation, sustained angiogenesis, tissue invasion and metastasis. Developments in molecular biology and genetics and the modern technology of microarrays have contributed to clarify the aetiology and pathogenesis of lung cancer and a great amount of studies showed more than 20 different genetic and epigenetic alterations that have been accumulated during the pathogenesis of the disease. The above alterations that lead to the accumulation of mutations are promoted by genomic instability through inhibition of apoptosis and alternative pathways.

Introduction

Two major types of lung cancer (LC) have been recorded, the small cell LC (SCLC) that is more rare with faster growth and more frequent development of metastases that already have been existed during the diagnosis procedure and the non-small cell LC (NSCLC) that is more common, whereas it has slow development. NSCLC is distinguished in squamous cell carcinoma, large cell carcinoma, adenocarcinoma (AC) (including broncho-alveolar carcinoma), adenosquamous and sarcomatoid carcinoma. LC histological types, squamous cell carcinoma, undifferentiated SCLC, undifferentiated large cell carcinoma, AC and adenosquamous, are also known as "bronchogenic cancer" or "bronchial carcinoma". This type is the most common and most deaths are caused by it [1]. Pathogenesis of the disease can be attributed to the accumulation of multiple molecular aberrations with the passing of time. Those molecular abnormalities lead to acquired cellularity capabilities that can be categorized into the following functional groups: self- sufficiency in growth signals due to mutations in proto-oncogenes, insensitivity to anti-growth signals as a result of mutations affecting the tumor suppressor genes, inflammatory micro-environment, evading apoptosis due to up-regulation of anti-apoptotic or down-regulation of pre-apoptotic molecules, limitless replicative potential due to telomerase activation, sustained angiogenesis, tissue invasion and metastasis [2].

Lung cancer molecular biology

Cigarette smoking accounts for about 85% of NSCLC cases and 98% of SCLC cases. Tobacco smoke contains more than 7,000 chemicals including over 70 known carcinogens that cause cancer such as nitrosamine ketones and polycyclic aromatic hydrocarbons (PAH) that are responsible for the genetic mutations due to the formation of a complex with DNA. That formation is induced by activation of the mentioned carcinogens metabolism, by metabolism of the above carcinogens by cytochrome P450, CYP family enzymes and glutathione-S-transferases (GSTs). In many LC signaling pathways the functions of the mentioned enzymes have been changed. Most of them are regulated by oncogenes that lead the cells to a malignant phenotype, uncontrolled proliferation and evasion of apoptosis [3].

The mentioned molecular changes concern oncogenes and tumor suppressor genes and can be occurred at the level of upstream or downstream gene regulation, due to changes in DNA sequence (point mutations), loss of heterozygosity (LOH), amplification of DNA segment or the loss or gain of an entire chromosome with a concomitant gene instability and changes in micro- satellite DNA [4,5]. Developments in Molecular Biology and Genetics as well as the modern Microarray technology have contributed to clarify the LC pathogenesis and a great amount of investigations have shown more than 20 different genetic and epigenetic alterations to be accumulated during the pathogenesis of LC as a clonal multistage process. The above conditions that lead to accumulation of mutations are promoted by genomic instability through inhibition of apoptosis and alternative pathways [6-8].

The most explored changes till now, are the inactivating mutations and loss of tumor suppressor genes and overexpression of oncogenes that induce tumor growth. Recently, it was observed the epigenetic inactivation of tumor suppressor genes due to promoter hypermethylation. Early clonal genetic abnormalities that are observed

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in pre-tumor bronchial epithelium that is damaged by smoking or several carcinogens have also been identified. Similarly, molecular differences have been described between SCLC and NSCLC cases and among tumors that are characterized by different clinical outcomes [9].

In SCLC patients, various tyrosine kinase receptors are overexpressed and involved in cell proliferation, migration and survival through alterations of the reactive oxygen species (ROS) and activation of downstream signal transduction molecules [10]. In addition, multiple chromosomes abnormalities that reflect genetic instability have been found in SCLC patients and in patients that suffer from other epithelial tumors. The majority of SCLC patients have shown deletions of multiple chromosome sites with specific losses on 3p, 5q,13q and 17p that contain tumor suppressor genes including p53. Analyses of comparative genomic hybridization have recorded a great number of the underlying gains in SCLC patients at regions 1p,2p, 9p, 5p, 8q and 19p. Those regions are responsible for encoding known oncogenes such as Myc and K Ras. SCLC cell lines showed amplification on 1p, 2p and 3q, with deletions of 18q and were associated with a more aggressive phenotype of the disease [11].

Chromosome 3p allele loss is a frequent event in 90% of SCLC patients and is considered an early event in LC pathogenesis [12]. Other loss regions that have been identified include the following regions 3p21.3,3p12, 3p14.2 and 3p24 [10]. Various genes in those loci play a role in tumor suppressive action and often lose their expression through epigenetic mechanisms. The tumor suppressor genes on the region 3p21 include RASSF1A, FUS1, SEMA3B and SEMA3F, whereas the FHIT (fragile histidine triade) gene is located at 3p14.2 locus, the region 3p24 contains the RARβ gene and all have an essential role in LC pathogenesis [13].

Oncogenes stimulate cell growth

Protein tyrosine kinases (PTKs) are vital intracellular signal transduction pathways regulators that mediate cellular growth and 'cell-to-cell' communication. Their action is normally controlled and regulated. Disturbances in signaling of PTKs derived from mutations and other genetic alterations contribute to the malignant transformation. A number of growth factors and their receptors are expressed by LC cells or the adjacent stromal cells and produce autocrine or paracrine growth stimulation loops that are encoded by proto-oncogenes and can be activated during the development of LC [9].

The overexpression of cell cycle protein regulators such as Cyclin D1 [14], Cyclin E [15] and Cyclin B1 [16], enhance cell proliferation, reduce potential of cell apoptosis, and are often found in NSCLC histological samples [9].

The epidermal growth factor receptor (EGFR), known as ErbB-1, is a member of a family of closely related proteins. EGFR belongs to the family of receptors with tyrosine kinase activity (RTKs), known as HER/ErbB family and consists of 4 known receptors: EGFR or HER1-4/ErbB1-4. Its activation by appropriate ligands, such as EFG, amphiregulin, heparin binding EGF, etc., results in the activation of the receptor's intracellular tyrosine kinase domain which undergoes auto-phosphorylation and can trigger a cascade of intracellular events. In particular, it leads directly to the activation of important intracellular signaling pathways such as Ras-Raf kinase (MEK), MAPK-kinase, Akt-RT3K kinase and mTOR-protein. The Ras-Raf-MEK-MAPK pathway regulates several cellular processes such as transcription, cell cycle progression at the G2/S phase and cell proliferation, whereas the PI3K pathway regulates anti-apoptotic mechanisms. EGFR is able to activate more pathways such as the STAT proteins and protein kinase C pathways, however, the effect of EGFR on the molecular level has not yet fully defined. In 50-80% of NSCLC cases it has been found that either the EGFR is overexpressed or an increased activity in the EGFR pathway activation has been found that can be attributed to the following mechanisms:

A. Increased protein levels of the receptor in the cancer cell membrane, with or without corresponding increase in levels of gene expression,

B. EGFR mutations that can result in continuous activation without the presence of the proper ligand,

C. Increased levels of proper ligands such as EGF or TFG-α (transforming growth factor alpha) [17].

The signaling pathway that is regulated by those receptors is complex and any disruption in its activation leads to processes that can induce carcinogenesis. Such processes are the inhibition of apoptosis, uncontrolled cell proliferation, insensitivity to anti-growth signals, angiogenesis, tissue infiltration and metastasis [18].

Another study showed that over-expression of EGFR was observed in 62% of NSCLC cases and it was often associated with poor prognosis and resistance to chemotherapeutic agents including cisplatin [19]. Deregulation of the EGFR signaling-pathway is observed in many types of cancer, whereas SCLC consists one of the solid tumors in which no overexpression of that receptor was observed. The HER-2/neu (ErbB-2) gene is located on chromosome 17p21 and encodes for a transmembrane glycoprotein (p185HER-2/neu) that is highly homologous to EGFR. HER-2/neu is overexpressed in approximately 30% of NSCLC cases, particularly in AC and is associated with multidrug resistance phenotype and a higher rate of metastases [9]. A point mutation that leads to substitution of a glutamic acid (Glu) for a valine (Val) at position 664 is usually found and contributes to malignant cell transformation. Alterations and amplifications of the HER-2/neu gene have been reported in NSCLC cases [18].

The Myc gene family encodes nuclear DNA binding proteins, c-, N- and L-Myc that function as transcription factors and thus regulate the cell proliferation, apoptosis and differentiation [20]. Those genes exhibit amplification in 15-30% of SCLC cases and in less rate in NSCLC cases [3,21]. Protein overexpression has been observed in SCLC cases due to Myc, activation that caused by gene amplification or transcriptional deregulation [22], which has been found in 18- 31% of NSCL cases [23] and is associated with reduced survival [10]. Myc phosphoproteins are located at the cell nucleus [24] and their transcriptional regulation is mediated by hetero- dimerization with proteins such as MAX, MAD or MX11 [25]. The Myc-MAX heterodimer binds to specific DNA sequences that are known as E-box elements in adjacent region of downstream target-gene and activates their transcription. On the other hand, the Myc-MAX complex suppresses the transcriptional activation. MAX has been found to interact with at least two proteins, MAD and MXI1leading to the transcription suppression, antagonizes Myc function and promotes cell differentiation [18]. The molecular abnormalities that are involved in Myc genes or their transcriptional deregulation were found to be significant molecular mechanisms in the pathogenesis of LC [15].

Gene amplification or gene-overexpression without amplification consist the most common abnormalities that involves Myc members in LC. Overexpression of c-Myc gene, with or without amplification, was found in 80-90% of SCLC cases, whereas the amplification of it is only found in10% of NSCLC samples. However, the overexpression of c-Myc gene without its amplification has been found in more than 50%

of NSCLC samples. c-Myc gene overexpression has been found to be a delayed event in LC pathogenesis in the majority of SCLC patients [24]. Cell lines derived from metastatic tumors showed a high incidence of c-Myc amplification and that finding may explain the relationship between c-Myc amplification and poor clinical prognosis [26].

The dominant RAS proto-oncogene is extremely important for signal transduction that promotes the cell proliferation. The RAS oncogene family includes the HRAS, KRAS 2 and NRAS genes, and consists a part of the signal transduction SOS-Ras-Raf-MAPK mitogenic cascade [2]. In malignant cells the point mutation in the RAS gene is able to make the RAS protein deficient regarding its inherent GTPase activity so that it is locked in the stimulated form continuously and sends signals that are able to stimulate cell proliferation in the nucleus [27].

Ras mutations are rare or absent in SCLC cases, however they can be found in 15-20% of NSCLC cases. Approximately 50% of the AC's show RAS mutations [28], and usually affect the KRAS codon 12, in 85% of cases, and rarely the HRAS codon 13 or the N RAS codon 61[25]. The majority, up to 70% of those mutations are G → T transformations induced by benzopyrene diethyloxide (BPDE), nitrosamines and other DNA-damaging agents that are contained in cigarette smoke. It is considered that this is the reason for the correlation between smoking history and the frequency of RAS mutations in NSCLC cases that are associated with poor prognosis [29].

Another pathway that is deregulated in LC is the ALK kinase (anaplastic lymphoma kinase fusion proteins), in which a fusion between the EML4 gene (echinoderm microtubule associated protein like 4) and the ALK gene is observed in 7% of NSCLC cases and this results in the activation of the ALK protein kinase domain. That finding is usually found in never smoked patients and may play a role in activation of the RAS [3,21].

The TTF1 pathway (thyroid transcription factor 1), which consists an important transcription factor that is necessary for the development of peripheral airways, when is inhibited by RNA- interference (RNAi) leads to inhibition of tumor growth and apoptosis [3,21].

Insensitivity to anti-growth signals: abnormalities in tumor suppressors genes

Tumor suppressor genes play a crucial role in cell anti-proliferative function and also participate in the cellular response to DNA damage and in subsequent redress procedures. A frequent loss of tumor suppressor genes during the LC pathogenesis and progression has been recorded, as well as in many epithelial cancers. Inactivation of tumor suppressor genes could be attributed to genetic or epigenetic mechanisms such as the loss of one allele from a chromosomal site, that is known as LOH and the inactivation of the second allele is caused by gene mutation or promoter hypermethylation. The most frequently chromosomal regions found to be affected by LOH in LC are 1p, 3p, 4p, 4q, 5q, 8p, 9p (p16 TSG locus), 9q, 10p, 10q, 13q (Rb-locus),15q,17p (p53 locus), 18q, 19p, Xp and Xq3. Chromosome 3p allele loss is one of the most frequent and earlier genetic events in LC pathogenesis and was found in 96% of cancers cases and 78% of pre-tumor bronchial epithelial lesions [30]. In previous studies in many cell lines and tumor samples have been observed high rates of LOH and an increased frequency of homozygous deletions. That observation indicates that few potentially tumor suppressor genes are located in this chromosome region [25]. In addition, the frequency and magnitude of 3p allele loss was associated with the severity of histopathological precancerous/pre-infiltrative lesions. A number of other candidate tumor suppressor genes are located on chromosome 3p and the loss of their alleles may possibly be the earliest acquired genetic abnormality in the pathogenesis of LC [9,31].

The FHIT gene has been shown to function as a tumor suppressor gene for most human cancer, is located at chromosome 3p14.2 and encodes for a protein called p FHIT. Loss of FHIT causes defective DNA replication, and leads to further DNA breaks. FHIT is specifically targeted and regulates death receptor (DR) genes. Pre-clinical studies have shown that the FHIT wild type infection in LC cells induces apoptosis [32].

Reduced expression of the pFHIT protein has been observed in 49% of the NSCLC samples, immunohistochemically, whereas homozygous deletions were present in 100% of the SCLC cases [33]. pFHIT expression is significantly decreased in a large number of early-stage NSCLC cases and in a number of pre-neoplastic lesions in chronic smokers. The relationship between cigarette smoking and expression of the pFHIT suggests a role for the gene and its association with smoking carcinogenesis [18]. It has been shown that the FHIT wild type restoration inhibits LC growth *in vitro* and tumorigenicity in nude (athymic) rats *in vivo* [25].

The RARβ gene (β -receptor of retinoic acid) is located at chromosome 3p24 and consists a candidate tumor suppressor gene. Low RARβ expression or a lack of RARβ expression, have been reported with high frequency in LC cell lines and primary lung tumors [25]. The mentioned finding appears to be derived from RARβ gene promoter abnormal methylation and was observed in 40% of primary SCLC cases. In another study, it was found that the RARβ gene is methylated in 72% of SCLC cases and leads to the loss of its expression. The RARβ gene regulates epithelial cell growth, tumor suppression and oncogenesis [34].

The tumor suppressor gene TP53 (p53) is located on the short arm of the chromosome 17 (17p13.1) and encodes for a nuclear protein that acts as a DNA-bound transcription factor that activates gene expression and is associated with cell cycle arrest in the G1 phase or in cell death in response to genotoxic stress. Its role is to preserve genome integrity by acting as the 'guardian' of the genome during cellular stress caused by DNA damage, hypoxia and activated oncogenes. It also prevents cells with damaged DNA from entering the G2 phase of the cell cycle by inhibition of CDC2 cyclin-dependent kinase that is required to enter mitosis. The ability of p53 to efficiently inhibit cell proliferation or to induce apoptosis can be suppressed by the HDM2 protein, the human homolog of the MDM2 protein. The HDM2 protein blocks the regulation of p53 target genes and enhances its cleavage by the proteasome [35]. On the other hand, p53 upregulates the expression of HDM2 via a direct connection and activation of the HDM2 promoter and thus p53 downregulates its own expression. This self-regulating loop maintains p53 in practically low or undetectable levels in normal cells [2]. The TP53 gene is more frequently mutated than any other gene in human genome and is one of the most significant events in LC. Its mutation is directly related to exposure to carcinogens and especially cigarette smoke [3,21].

Nonsense mutations, mainly G → T transpositions that are concentrated in the middle of the gene at codons 157, 245, 248 and 273 are able to abolish the activity of this protein as a suppressor protein and extend half-life of p53 mutant protein which can readily be detected immunohistochemically. The p53 gene mutations in LC patients have been extensively investigated and was found that p53 is inactivated in

75% of SCLC cases and in 50% of NSCLC cases [18,33]. Inactivating mutations have been observed in 90% of the SCLC cases, most of which are non- sense mutations in the DNA binding domain and a smaller number are homozygous deletions [12]. In 40-70% of SCLC cases has been recorded an abnormal expression of p53 protein [33], whereas its mutations are related to cigarette smoking, especially the GC →TA transformation is caused by carcinogenic benzo(a)pyrene of tobacco [10].

Mutations at codon 157 are unique in LC cases whereas those at codons 248 and 273, especially 'hot spots' mutations, are also present in other cancers, such as in colon, liver and prostate. Non- smokers with LC show a totally different and random grouping of p53 mutations [24]. The tumor suppressor gene Rb is located at chromosome 13q14 and its product, a nuclear phosphoprotein was first found in the childhood retinoblastoma. Rb protein co-operates with p53 in the cell cycle regulation and control, in the transcriptional level and in the balance between cell differentiation and proliferation. The Rb protein phosphorylation status and its interaction with the transcription factor E2F play a critical role in the regulation for the G0/G1transition [36].

Abnormalities of the Rb gene in LC cases include antisense mutations, pathogenic splice variants and chromosome deletions. More than 90% of SCLC cases and 15-30% of NSCLC cases show abnormal or non-expression of the Rb protein [24].

Although the Rb protein plays an important role in the LC pathogenesis , its status has no prognostic significance in NSCLC patients [18]. The phosphatase and tensin homolog (PTEN) gene is located on chromosome 10q23 and encodes for a lipid phosphatase which dephosphorylates PIP3 and exhibits tumor suppression activity *in vitro* and *in vivo*. Mutations or deletions of that gene have been found in a few LC cell lines and tumor samples [25].

The transforming growth factor-β (TGF-β) is a multifunctional protein that inhibits the proliferation of most epithelial cells by binding to a great number of cell receptors. It consists an inhibitor-checkpoint that is involved in the cell cycle regulation, prompting the cells to stop their proliferation during the G1-phase of the cell cycle. Reduced expression levels of TGF-β were found in NSCLC samples by the use of immunocytochemical methods [24].

Deleted in malignant brain tumors 1 gene (DMBT1) is a candidate tumor suppressor gene that is located at chromosome 10q25-26 and is often downregulated and occasionally homogeneously deleted in LC [25].

P16INK4 (or CDKNA2) is a tumor suppressor gene that is located at chromosome 9p21 and encodes for two proteins that are translated by alternative mRNA splicing, α-transcription that is translated into p16 (p16INK4) and β-transcription that is translated into p14ARF-protein. Loss of p14ARF or p53, that are common genetic alterations in LC, allows for an accumulation of c-Myc and, therefore, for cell proliferation and transformation. The p14ARF protein appears to bridge the gap between oncogenic signals and p53, according to which the p14ARF-mediated activation would be critical to lead the cell to apoptosis [24, 35].

Expression of p16INK4 gene in NSCLC cases is often altered due to abnormalities of promoter methylation , in 25% of cases, homozygous deletions or point mutations in 10-40% [25].

It has been found that the disorders in both pathways, p16/Rb and p53, are essential for the enhanced proliferation in NSCLC cell lines. A reverse relationship between p16 and Rb in LC has been observed in which the Rb gene is mutated and p16 gene is intact in SCLC cases, whereas the p16 expression is disrupted and the Rb gene is usually intact in NSCLC cases [24]. In addition, in SCLC cases, the most common abnormality in the p16INK4A-Cyclin D1-CDK4-Rb pathway that mediates the transition from phase G1 to phase S of the cell cycle concerns the Rb gene. The types of the Rb gene mutations include deletions, antisense mutations and splicing abnormalities [37]. Complete loss of the gene or a mutant form of it have been found in more than 90% of SCLC cases [38].

p19ARF binds to MDM2-p53 and prevents the p53cleavage. Loss of p19ARF is more common in lung tumors with neuroendocrine features [25,35].

Methylation-associated inactivation of RASSF1A gene has been observed in many human malignancies [13]. That gene encodes for a protein similar to RAS effector proteins and is in- activated in more than 90% of SCLC cases [14]. RASSF1A is also involved in cell cycle path- ways, apoptosis and microtubule stabilization [39].

Loss of FUS1 protein expression has been found in 100% of SCLC cases [40]. The wild type of the gene induces G1 phase arrest and apoptosis [12].

Evading apoptosis

Apoptosis or programmed cell death is a genetically controlled process that is essential for the remodeling of tissues during embryogenesis and for maintaining of the homeostatic balance of the number of cells during their function. The deregulation of the cell death pathways is involved in the tumor initiation, development and resistance to treatment in many human cancers and consists one of the main features of cancer [2,41].

Main genes that regulate the apoptosis process are p53 tumor suppressor gene and the Bcl-2 gene family. The members of the Bcl-2 family are the main regulators of the apoptotic process whereas caspases are the main effectors [42,43]. The Bcl-2 family includes anti-(bcl-2, bcl-X, mcl-X) and pro-apoptotic proteins (bax, bak, bad) that regulate the cell death and mechanisms such as apoptosis, necrosis and autophagy [44], whereas alterations in its expression lead to the pathogenesis and progression of malignant neoplasms [45]. BAX protein is associated with Bcl- 2, promotes apoptosis and constitutes a downstream transcriptional target of p53. The Bcl-2 protein heterodimerizes with BAX and inhibits apoptosis. Tumor cells often escape apoptosis, as a normal physiological response in case those cells are exposed to cellular lesions and lesions in DNA. Anti-apoptotic Bcl-2 proteins are typically overexpressed in many malignancies and their expression is often related to drug sensitivity [46,47].

Overexpression of Bcl-2, that is detected by immunohistochemistry, was found in 75-95% of SCLC cases, in 25-30% of squamous cell cancers cases and in 10% of AC cases [48,49]. The significantly higher frequency of Bcl-2 overexpression in SCLC cases is unexpected as these tumors are more sensitive to chemotherapeutic agents that induce an apoptotic response. Interestingly, BAX and Bcl-2 proteins expression is inversely related to neuroendocrine cancers, whereas high Bcl-2 and low BAX expression have been found in the majority of SCLC cases that appear inadequate in identifying p53 expression [9].

The importance of the Bcl-2 protein expression in LC for overall survival is ambiguous but the Bcl-2 expression was found to be associated with better prognosis in patients with NSCLC that may be associated with a lower level of tumor vascularization [18,50].

The nuclear factor NF-kB is activated by inflammatory cytokines (TNF-α, IL-1β, IFN-γ) and bacterial products (LPS), consists an important effector of the inflammatory response and is involved in the evasion of programmed cell death. That factor is activated by the smoke components, whereas it is also activated in epithelial cells and macrophages in Chronic Obstructive Pulmonary Disease (COPD) patients, it shows anti-apoptotic action by interaction with Bcl-2 and Bcl-XI factors and is associated with chronic inflammation, such as COPD, and carcinogenesis of the respiratory tract. Its activation by chemical carcinogens concerns the epithelial cells and macrophages as mentioned, whereas its inhibition leads to a decrease in chemical carcinogenesis by 58% [51].

Limitless replicative potential due to telomerase activation

Telomeres are specific chromatin structures at the end of each chromosome that are used as protective caps and play a role in maintaining of their integrity, as are able to suppress reversibly the transcription of adjacent genes and to prevent the 'end-to-end' fusion or the cleavage of the chromosomes [52].

Because of the inability of conventional DNA polymerases to replicate 5′-terminal of DNA, the telomeres are shortened in normal human somatic cells during cell division , a phenomenon known as a 'end-replication-problem'. That shortening does not lead to loss of essential genes as each chromosome is covered by telomeres. The human telomere consists of highly repetitive sequences (TTAGGG) [53] and it has been estimated that 50-100 bp are lost per cell division cycle [54]. It has also been observed that human cells have a potential to undergo a value of approximately 50-70 divisions. After that limit the cell growth is interrupted and enter a phase of senescence. Telomere maintenance requires complex interactions between proteins, telomeric DNA and other cellular factors. Telomere integrity is also essential for chromosomal stability and telomere shortening facilitates the progression of cancer cells as leads to 'end-to-end' chromosome fusions and aneuploidy development. Telomerase inhibition in immortal cancer cell lines with application of genetic or pharmaceutical methods leads to telomere shortening and ultimately can stop cell proliferation [55].

Telomerase is a ribonucleoprotein enzyme complex that prolongs and maintains chromosome ends- telomeres of the eukaryotic chromosomes using one native RNA molecule as a template and thus extends the number of cell divisions [56]. In other words, it is a RNA-dependent-DNA- polymerase that can compensate for the loss of those DNA sequences by synthesizing telomeric replicates in cells that are able to be divided. A catalytic subunit, telomerase reverse transcriptase (hTERT) with the telomerase RNA component (TERC) comprises the most important unit of the telomerase complex [57].

Telomerase activity is absent in most normal cells in adults but increases during tumor growth [58]. In differentiated cells telomerase activity is silent. Cells acquire the immortal phenotype by compensating for loss of telomeric repeat by reactivation of telomerase [59], leading to continuous cell division. Given that more than 90% of human neoplastic cells show an increased telomerase activity, it is accepted that this is one of the main features of cancer, is quite common and related with the molecular abnormalities in cancer. Telomerase expression in malignant tumors determines the ability for unlimited proliferation and hence the immortality of those cells. High telomerase activity was detected almost 100% of SCLC cases and 80% of NSCLC samples using PCR technique. In addition, high telomerase activity in primary NSCLC cases was found to be correlated with elevated cell proliferation rates and advanced pathological stage [60]. It has also been found that more than 98% of SCLC cases showed upregulation of telomerase activity [57,61].

Recently, shortening of the telomere was found to be an early molecular abnormality in the broncho-epithelial carcinogenesis preceding the expression of telomerase and the inactivation of p53/Rb that observed in the majority of pre-invasive lesions [62].

Based on the fact that telomerase activity is associated with malignant growth, it can be used as an indicator for LC detection as well as an important target for new therapeutic approaches [25].

Tumor angiogenesis

The development of new blood vessels, neovascularization, is necessary for tumor development beyond 3,00 mm in diameter and is critical to the metastatic ability. Different inducers and inhibitors regulate the endothelial cell proliferation and migration as are involved in the process of angiogenesis. Growth factors that have been found to stimulate angiogenesis are the vascular endothelial growth factor (VEGF),the fibroblast growth factor (b FGF), the platelet-derived endothelial cell factor (PDEGF) and the platelet-derived growth factor (PDGF) [9,25].

VEGF is a family of factors that consists of VEGF-A, B, C, D and F factors and VEGFR 1-3 receptors. The VEGF signaling pathway leads to increased proliferation, migration and invasion of endothelial cells, mediating tumor angiogenesis. Autocrine VEGF/VEGFR signaling pathway regulates cell proliferation and metastases development [63].

High levels of VEGF have been found in SCLC patients and were related to the tumor staging, progression , resistance to chemotherapy and poor prognosis. SCLC cells express VEGFR-1 and -2 and are involved in tumor growth and infiltration [64].

The products of angiogenic factors affect the clinical prognosis in LC patients, i.e VEGF plasma levels are related to the degree of vascularization in NSCLC cases, whereas VEGF expression was found to be associated with a decreased overall- and disease-free survival in NSCLC patients [65].

Basic fibroblast growth factor (bFGF or FGF-2) shows a biological role in SCLC cases [64]. Elevated levels of bFGF in the serum of SCLC patients are associated with poor prognosis and active angiogenesis [66]. In addition, bFGF promotes the development of SCLC cells and leads to resistance to chemotherapeutic drugs [67,68]. Immunochemical studies have shown that bFGF is a prognostic marker in LC patients as the 5-year survival rate was significantly lower for bFGF positive patients, whereas its aggressive clinical behavior was associated with upregulation of PDGF [25].

Tissue infiltration and metastasis

The molecular mechanisms that lead the cells of the primary LC to infiltrate the adjacent tissues and spread to remote regions and organs, remain unknown [9]. This process involves de- gradation of the basement membrane, infiltration of the environmental layer, blood or lymphatic vessels, developmental ability without attachment, angiogenesis, cell proliferation and migration [2]. A small number of different genes as well as their protein products have been identified as important for the process of tissue infiltration and the metastatic ability of neoplastic cells. E- Cadherin is a cell adhesion molecule that is expressed mainly by epithelial cells. During the pathogenesis of most epithelial cancers, its

function is lost by the mutational inactivation of its genes or β-Catenin genes as well as by the transcriptional suppression or the enhanced proteo- lysis. That process leads to a decreased, E-Cadherin-mediated 'cell-to-cell' adhesion and allows malignant tumor cells to infiltrate the tissues and to enter the blood or lymphatic vessels [69]. Thus, E-Cadherin gene is known as a "infiltration suppressor gene" [70] and it was also found that the loss of E-Cadherin in LC cases was associated with increased metastatic ability [71].

The basement membrane and the extracellular matrix can be degraded by proteases and that process is of a vital importance for local infiltration and vascular or lymphatic metastasis. The matrix metalloproteinases (MMPs) are one of the main families of proteinases and especially are calcium-dependent zinc containing endopeptidases which facilitate infiltration, the ability to metastasize and the tumor-associated angiogenesis. In contrast, inhibitors of matrix metalloproteinases (TIMPS) has been found to inhibit tumor growth and its spreading in pre-clinical models. Therefore it remains unknown why all LC cases do not express MMPs whereas there have been found conflicting options regarding the predictive significance of their expression in LC [72].

It was found that collapsin response mediator protein-1 (CRMP-1), a protein that can mediate the effect of collapsines, has decreased expression in more aggressive and metastatic LC samples [73]. That down-regulation can enhance the ability of cell migration that is important for the process of metastasis. Those CRMP-proteins and other members of the collapsin/semaphorin proteins family can regulate cell mobility [74].

Laminins and integrins are proteins involved in infiltration of adjacent tissue through the basement membrane and are responsible for LC widespread . The decreased expression of laminin α- chains (α3 and α5) in pulmonary neoplastic tissue can lead to the fragmentation of the basement membrane that is required for the infiltration of cancer cells [75].

Alterations in integrin's expression have been found in metastatic cells in many human neoplasms as well as in LC [2,76]. Recently it was found that the LAMB3 gene, that encodes for laminin β3 chain, a component of laminin-5, was only expressed in NSCLC cells and not in SCLC cells. In the same study, α6β4 integrin, the specific binding receptor of laminin- 5, was expressed only in NSCLC cells, but not in SCLC cells. This observation suggests that laminin-5 may be has a critical role in NSCLC development [77].

Tyrosine kinase receptors (RTKs) - growth factors in SCLC

The c-Kit receptor is a member of the PDGF/c-Kit RTKs family. Following ligand binding, that is a stem cell factor (SCF), cell growth and differentiation begins with the activation of JAK/STAT, PI3K and MAP-kinase pathways [62] and thereby contribute to the pathogenesis of SCLC [78]. c-Kit expression has been found in 79-88% of the SCLC cell lines, whereas both c- Kit and stem-cell factor (SCF) expressions have been found in 57-76% of SCLC cell lines [10]. c-Met RTK is activated by its ligand, hepatocyte growth factor/scatter factor (HGF/SF) and activates cationic molecules such as Grb 2 (growth factor receptor-bound protein 2), p85 sub- unit of PI3K, STAT3 and Gab1 (Grb 2-associated binder-1). Overexpression and amplification of c-Met have been found in LC cells, whereas higher levels of HGF are associated with poor prognosis [10, 62]. HGF/c-Met pathway promotes more aggressive tumor type in SCLC and modulation of c-Met/HGF pathway results in changes in cell mobility and migration [79,80].

IGF-1receptor (Insulin-like growth factor-1 receptor) is a member of the RTKs superfamily and is activated by IGF-1 and -2 ligands, induces mitogen signal transduction and is associated with anti-apoptotic and transformation activities. The IGF-1R receptor and its ligand are expressed at high levels in SCLC cell lines, whereas more than 95% of SCLC cases, IGF-1protein levels have been found elevated [10,62]. In addition, the IGF-1R receptor activates the PI3K-AKT signaling pathway in SCLC cases and contributes to the development of the disease as well as the resistance to chemotherapy [62]. The fibroblast growth factor receptor (FGFR) is a member of the RTKs family that contains 4 different isoforms (FGFR1-4). Upon binding to fibroblast growth factors (FGFs) the receptor interacts with multiple signaling proteins and activates the Ras/Raf/MEK/Erk 1, 2 and PI3K-AKT signaling pathways [81] (Table 1).

Epigenetic alterations in LC

The term epigenetic alterations refers to molecular mechanisms that regulate gene expression without alteration in the DNA sequence and includes histone modification and DNA methylation that is the main epigenetic mechanism. DNA methylation is essential for normal development and gene regulation. In the pathogenesis of cancer may play a critical role 3 forms of abnormal methylation, namely hypermethylation of tumor suppressor genes, hypomethylation and the fact that methylation can regulate the expression of some micro-RNAs (mi-RNAs). In LC patients, it was found that many genes are not expressed due to methylation of the primer. Methylation is an early event in LC pathogenesis and its detection in the sputum of patients with dysplasia can be considered as a high-risk indicator for the appearance of the disease. In addition, detection of DNA methylation may be a prognostic factor for early recurrence in NSCLC stage 1 [82].

Another molecular mechanism are mi-RNAs, a small non-coding RNA molecule that it contains about 22 nucleotides and regulate gene expression as is involved in activity of specific mi-RNA targets by interactions among bases. Many mi-RNAs have a specific expression depending on tissue type. Their expression is often dysfunctional in many cancers, including LC, whereas they can play a biomarker role as they are released into blood circulation by the cancer cells. Interestingly, some mi-RNA may function as oncogenes or tumor suppressors genes [83] (Table 2).

The role of NOTCH in LC

It is a highly conserved developmental pathway that is significantly involved in tissue formation, cell differentiation and morphogenesis. Indications have shown that it plays an important role in several malignancies and can function as an oncogene or tumor suppressor gene depending on the cell type. Notch pathway is essential for the cell functions in tissues such as skin, pancreas and systems such central nervous, vascular, hematopoietic and muscular. In most tissues inhibits the initial cell differentiation by causing a second differentiation or by maintaining the cell in a non-undifferentiated status. It has been described as the guardian against differentiation [84].

The Notch family is transmembrane proteins that have dual function, as membrane receptors and as transcription factors. In mammals, the Notch family has 4 receptors and 5 ligands for those receptors, Delta 1, 3, 4 and Jagged 1 and 2. When a ligand binds to its receptor, the pathway is activated and TACE metalloproteinase (tumor necrosis factor a converting enzyme) and the γ-secretase complex degrade the receptor by proteolysis. The part of the receptor derived from the activity of γ-secretase releases the intracellular part of Notch (NICD)

Table 1. Signaling pathways and main types of genes involved in LC

Cell Signaling Pathway	Adenocarcinoma Genes involved (%)	Squamous Carcinoma Genes involved (%)	Small Cell Carcinoma Genes involved (%)
TP53	50 P53, MDM2	80 TP53	80-90 TP53
RAS/RAF	25 KRAS, NF1, BRAF, NRAS	22 NF1, KRAS, HRAS, NRAS, RASA1, BRAF	
PI3K/AKT	10-12 PIK3CA, PTEN, AKT1	59 PIK3CA, PTEN, AKT1-3, TSC1-2	10 PTEN
MYC	30 MYC		16-30 MYC, MYCN, MYCL
RTK	50 EGFR, ALK, RET, ERBB2, ROS, MET	27 EGFR, FGFR1-3, ERBB2/3, DDR2	6 FGFR1
RB1/CDKNA2	15-20 CDKNA2	79 CDKNA2, RB1	100 RB1, CCNE1
Epigenetic regulation	22 SMARCA4, ARID1A, SETD2	20 MLL2	19 EP300, MLL, CREBBP
Response to oxidative stress	10 KEAP 1	KEAP1, CUL3, NRF2	
Developmental pathway	20 NKX2.1/TTF1	44 SOX2, NOTCH1/2, ASCL4,FOXP1, TP63	20 EPHA7, SLIT2

Table 2. The main somatic genetic alterations in LC

Gene	Signaling Pathway	Aberration	Adenocarcinoma (%)	Squamous Carcinoma (%)	Small Cell Carcinoma (%)
EGFR	RTK	Mutation Amplification	20-30 >20	Rare 7	
ROS	RTK	Mutation	1.5		
MET	RTK	Mutation, Amplification after treatment with EGFR inhibitor	5 20		
ERBB2	RTK	Mutation, Amplification	2-4 5-10		
ERBB3	RTK	Mutation		2	
ALK	RTK	Fusion with EML4, etc.	3-13		
RET	RTK	Translocation with KIF5B, etc.	1-2		
FGFR1	RTK	Amplification	1-3	22	6
IGF1R	RTK	Overexpression			95
LKB1	LKB1/AM PK	Mutation	15-30	2	
CDKNA2/p16INK4	RB1/CDK	Deletions, Silencing, Mutation	>20	72	
RB1	RB1/CDK	Mutation	Rare	7	100
CCNE1	RB1/CDK	Gain of copy	12		
KRAS	RAS	Mutation	30	5	
NF1	RAS	Mutation	8-10	11	
HRAS	RAS	Mutation		3	
NRAS	RAS	Mutation	<1	<1	
RASA1	RAS	Mutation		4	
BRAF	RAF	Mutation	6	4	
PIK3CA	PI3K	Mutation	Rare	16	
PTEN	PI3K	Deletion	Rare	8	
AKT1,2,3	PI3K		Rare	16 (AKT3) 20 all genes	
TSC1,2	PI3K			6	
TP53	TP53	Mutation, Gene copy number loss	50 20	80	70
MDM2	TP53	Amplification	20		
MYC	Regulator of transcription	Amplification	31	Rare	16
MYCN	Regulator of transcription	Amplification			
MYCL	Regulator of transcription	Amplification			
SOX2	Developmental pathway	Amplification, Overexpression		21	
TP63	Developmental pathway	Amplification, Overexpression		16	
NOTCH1	Developmental pathway	Mutation		8	
NOTCH2	Developmental pathway	Mutation		5	

into the cytoplasm which then is transferred from the cytoplasm to the nucleus and activates the transcription of target genes. The main target genes of that signaling pathway are two families of transcription factors, Hes and Herp, whereas other target genes are Cyclin D1, p21, NF-kB, preTa, GATA3, NRAPP, c-Myc and Deltex1 [85].

Notch pathway controls several stages of cell differentiation in different tissues and may be involved in the development of different types of tumors. Several investigations have indicated that is associated with lung, ovarian, breast cancer and leukemias. It is also closely linked to the ras signaling pathway and its activation is possible a result of oncogenesis mediated by the ras pathway. Once the Notch pathway is activated by the ras pathway it can be inversely triggered the ras-raf-mek-MAPK pathway that consists a part of ras signaling pathway [86].

Notch signaling pathway is also critical for normal embryonic development. Given that tumori- genesis and organogenesis share the same mechanisms it is supposed that the cell signaling path- ways that are associated with human development and growth such as Notch, Wnt and Hedgehog play a role in the development and proliferation of cancer cells. Highly aggressive cancer cells have shown that they have many features of embryonic stem cells and use the Notch signaling pathway for its survival [87].

Dysfunction of Notch pathway is associated with many types of cancer and it has been shown that may has an oncogenic or tumor-suppressive action, depending on the tissue or the location of the organ expressed. The way in which the activation of the pathway itself leads to two adverse effects in different cell types remains unknown. A possible interpretation for that paradoxical behavior is that the Notch pathway activates/inactivates specific genes depending on the cell type or the signaling pathways which are the ones that determine the final effect of the Notch pathway on the cell. In conclusion, this pathway plays a critical role in maintaining a balance in the cell proliferation, survival, apoptosis and differentiation that affect the development and function of many organs. Dysfunction of that pathway also prevents the differentiation and can lead to malignant transformation [85,86].

Regarding the role of that signaling pathway in LC, its function can follow two models, the one that is related to the adjacent cells communication and its different evolution and the second that is associated with the autonomic activity of the Notch receptors. In NSCLC cells, it has been shown that Notch 3 promotes tumor growth [88].

Another study showed that Notch 1 is under expressed in NSCLC cases and the expression of the self-active Notch 1 in AC cells causes cell death. It is interesting that Notch1 under hypoxic conditions is overexposed, which is important for the survival of cells in LC patients and still shows that the concentration of oxygen determines the biological activity of Notch 1 in LC. A similar observation concerns the Notch 1 and 3 expression in the cell line A549 and SPC-1 of the AC. Overexpression of the Notch intracellular domain (NICD) inhibits the growth of the AC A549 cells *in vitro* by inducing cell cycle arrest and this leads to suppression of tumor growth [89-91].Those observations suggest that the Notch pathway may have a tumor suppressor activity in AC cells. By comparison, in SCLC cells Notch1 and 2 are under expressed, whereas its overexpression inhibits the development of cancer cells.

Signaling pathways in LC

PI3-K/AKT/mTOR pathway

Phosphoinositide-3-kinases (PI3Ks) are a family of lipid protein kinases that regulate cellular functions such as cell proliferation, survival, mobility, adhesion and differentiation. When PI3Ks are activated by RTKs and G-protein coupled- receptors translate signals from multiple growth factors and cytokines into intracellular signals by the formation of phospholipids that activate cationic effector pathways, including AKT (protein kinase B), a Ser/Thr protein kinase [10].

One of the main cationic effectors of the PI3-K/AKT pathway is mTOR (mammalian target of rapamycin), that phosphorylates and activates the ribosomal protein S6-kinase (S6K1) and the eukaryotic translation initiation factor 4E-linked protein 1 (4EBP1-eukaryotic translation initiation factor 4E-binding protein), that regulates the protein synthesis [92]. PTEN is a tumor suppressor gene and the major negative regulator of the above signaling path- way as antagonizes PI3K [93].

The PI3-K/AKT/mTOR pathway is disrupted in SCLC cells, as the tumor cells show active mutations in PI3K and PTEN [94]. AKT is phosphorylated in 70% of SCLC cases [62], whereas protein expression of mTOR and S6K1 and phosphorylated 4EBP1 are at elevated levels in SCLC cells compared to alveolar epithelial type II cells. Such alterations lead to tumor growth, survival and resistance to chemotherapy in SCLC cases [95].

Developmental pathways

Developmental pathways such as Hedgehog, Notch and Wnt that regulate the self-renewal of stem cells when they are abnormally activated, can cause a neoplastic proliferation, representing an early event in oncogenesis [96].

NSLC displays a characteristic neuroendocrine phenotype, expressing neuronal and endocrine markers, such as synaptophysin, chromogranin and CD-56 [91]. Neuroendocrine cells are the first recognizable differentiated cells in the airways epithelium during lung development [88].

Such differentiation of neuroendocrine cells from the underlying endoderm is regulated by the Notch pathway and abnormalities of those cells can lead to extend that cellular compartment. There is evidence that SCLC is the most undifferentiated epithelial tumor of the airways and its features are similar to the ones of the early formation of the lung [97]. SCLC development depends on abnormalities in Notch and activation of Hedgehog pathway. Targeting of those pathways can lead to the elimination of SCLC cloned cells and to more lasting effects of treatment [98].

Sonic Hedgehog pathway plays an essential role during early lung development and in epithelial- mesenchymal interactions [99,100]. Three well known ligands of that pathway in human organs have been described, Sonic Hedgehog (SHh), Indian Hedgehog (IHh) and Desert Hedgehog (DHh). The signal transduction cascade begins with the binding of Hh in the Pattched-1 receptor (Ptch-1), a transmembrane protein of 12 segments. In absence of Hh ligand, Ptch-1 inhibits the seven transmembrane proteins Smoothened (Smo) and leads to inactivation of that pathway. However, the connection of Hh to Ptch-1 leads to the inhibition of Smo release and then a protein complex is activated and leads to downstream transcription of Hh target in the nucleus, including Gli-1and Ptch-1.The active Hedgehog pathway leads to the extension of an intra- epithelial cell population during the regeneration of the airways after injury [100]. Activation of the Hedgehog pathway in that intraepithelial cell population predominates directly in the development of the neuroendocrine cells of the airways [101]. In SCLC cases has been observed a ligand-dependent activation of the Hedgehog pathway, whereas the adjacent cells express SHh [96,98,100].

The Notch pathway regulates the growth and differentiation of cells in various frames [10,102]. The most important function is to maintain non differentiated pluripotent cells in tissues [103] and plays a critical role in regulation of the airway epithelium development and is especially involved in neuroendocrine or non-neuroendocrine cell differentiation [88].

Overexpression of Notch receptors cause cell cycle arrest and inhibit the development of SCLC. Therefore, activation of the Notch pathway could be an effective strategy for SCLC patients [91].

The Wnt pathway includes proteins, a family of secretory molecules with various types of expression and a series of functions such as cell proliferation, differentiation, survival, apoptosis and cell mobility [104]. Three Wnt signaling pathways have been characterized. The first is the normal signaling and is activated by binding Fz (Frizzled) ligand and LDL-protein-related receptor (LRP) and leads to stabilization of the cytosol and nuclear translocation of β-Catenin for target gene expression (Wnt/β-Catenin pathway) [105]. The second pathway involves the activation of the calcium/calmodulin-dependent protein kinase II and protein kinase C which is a non-canonical pathway. The third pathway, known as non-canonical planar cell polarity path- way, acts through small GTP-ases such as RhoaA and Jun Kinase (JNK) and participates in re- arrangements of cytoskeleton and cell polarity [106].

During lung morphogenesis, is required a special Wnt signaling for normal epithelium- mesenchymal interactions. When the Wnt pathways are deregulated, poisonous effects can be appeared. In the adult lung all the components of Wnt signaling are present in detectable levels. Broncho-alveolar stem cells coexpress Clara and protein markers of epithelial cells are maintained and activated by the Wnt signaling[107]. When the bronchial cells are exposed to cigarette smoke, the Wnt pathway is activated and leads to proliferation and tumor growth [108]. In NSCLC samples, Wnt molecules are expressed differently by upregulation of Wnt proteins, such as Wnt 1 and 2, and decreased expression of Wnt modulators, such as WIF1 (Wnt inhibitory factor 1) [107].

Intracellular molecule chaperones-heat shock protein (HSP-90)

Heat shock proteins are a family of highly homologous chaperone proteins and are involved in protein maturation, proteins transposition along the membranes, proteins quality control in endoplasmic reticulum and normal protein mobility [109]. The Hsp-90 is an expressed cell protein, that is increased in tumor cells, under stress conditions due to the presence of mutant and deregulated proteins, oxidative damage, hypoxia or low nutrient environment [110].

Those proteins also include many oncogenic members such as AKT, MET, bcl-2, telomerase, survivin and Apaf-1, thereby can promote cell survival, growth and metastases as are able to allow for continuous protein translation and cell proliferation [111]. Thus, inhibition of Hsp-90 may be influenced the function of the correspondence chaperone in those malignancies and transformed cells, leading to disruption of oncogenes and multiple signaling pathways [110]. In SCLC cases was observed an overexpression of anti-apoptotic proteins and decrease in pre- apoptotic molecules, leading to deregulation of apoptosis. Hsp-90 is a major inhibitor of apoptosis in SCLC cases and differs from other cellular systems [111]. Pre-clinical studies have shown that Hsp-90 inhibition in SCLC cells releases Apaf-1that can leads to the formation of the Apaf-1 and caspase-9 apoptotic complex. That complex causes significant apoptosis only after the activation by Cyt C that is released from mitochondria, and is activated by the simultaneous inactivation of AKT by the inhibition of Hsp-90. When the AKT is downgraded, the BAD is dephosphorylated. Then the BAD heterodimerizes with selective antiapoptotic Bcl-2 family members or activates pre-apoptotic Bax and Bak proteins causing mitochondrial depolarization [96,111]. It is obvious that Hsp-90 regulates apoptosis in SCLC cases as a negative regulator of Apaf-1 and by controlling survival pathways PI3K-AKT [111].

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